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Volume 1

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The Proceedings are organized into eight main sections corresponding to the format of the Symposium and addressing Physical and Chemical Characteristics of Diesel Emissions, In Vitro Carcinogenic and Mutagenic Effects of Diesel Emissions and Components, Biochemical and Metabolic Effects, Toxicological Effects of Inhaled Diesel Emissions, Mutagenic and Carcinogenic Potency of Extracts of Diesel and Related Environmental Emissions, Mutagenicity of Inhaled Diesel Emissions, Carcinogenic Effects of Exposure to Diesel Emissions, Epidemiological Studies, and lastly a panal discussion on Health Risk Assessment of Diesel Emissions.

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Health Effects Research Laboratory

Edited By

W. E. Pepelko, R. M. Danner, N. A. Clarke

HEALTH EFFECTS RESEARCH LABORATORY
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U. S. ENVIRONMENTAL PROTECTION AGENCY
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FOREWORD

The U.S. Environmental Protection Agency was created because of increasing public and government concern about the dangers of pollution to the health and welfare of the American people. Noxious air, foul water, and spoiled land are tragic testimony to the deterioration of our national environment. The complexity of that environment and the interplay between its components require a concentrated and integrated attack on the problem.

Research and development is that necessary first step in problem solution and it involves defining the problem, measuring its impact, and searching for solutions. The primary mission of the Health Effects Research Laboratory in Cincinnati is to provide a sound health effects data base in support of the regulatory activities of the EPA. To this end, HERL conducts a research program to identify, characterize, and quantitate harmful effects of pollutants that may result from exposure to chemical, physical, or biological agents found in the environment. In addition to the valuable health information generated by these activities, new research techniques and methods are being developed that contribute to a better understanding of human biochemical and physiological functions, and how these functions are altered by low-level insults.

Together with the problem posed by air pollution from automotive emissions, the Environmental Protection Agency inherited a program, designed to define and measure the impact of that problem, initiated in 1961 within the Public Health Service's Division of Air Pollution in Cincinnati, Ohio. Part of that program remains with the Agency's Health Effects Research Laboratory in Cincinnati. It is therefore appropriate that the first International Symposium on the Health Effects of Diesel Emissions should have been held in Cincinnati and that it should have been sponsored by the Health Effects Research Laboratory.

This report of the Proceedings represents an attempt to assemble current knowledge relevant to an assessment of the potential impact upon human health and welfare of diesel-powered light-duty vehicles. With a better understanding of the health effects, appropriate control measures can be introduced as necessary.

R. J. Garner
Director
Health Effects Research Laboratory

PREFACE

The Environmental Protection Agency, under the Clean Air Act, is charged with the responsibility for regulating emissions from new motor vehicles. It has been estimated that ten to 25 percent of new U.S. passenger cars could be diesel powered by 1985, since such engines offer about a 25 percent improvement in fuel savings over comparable vehicles powered by gasoline engines. The various gases in diesel exhaust are similar to those emitted by gasoline engines; however, the particles in diesel exhaust are quite different in composition and quantity from those in gasoline engine exhaust - even a properly tuned diesel engine will emit 30 to 100 times more particulate matter than a comparable gasoline engine with a catalytic converter. These diesel engine particulates are basically a carbonaceous material with mainly high molecular weight organic chemicals adsorbed to them. Additionally these particulates, because of their small size, are respirable and are known to penetrate deeply into the lungs. Because of these facts, there is an obvious need for well designed studies on the health effects of diesel emissions. These studies will, hopefully, provide data useful in making risk assessments and provide the regulators with appropriate criteria for establishment of scientifically based standards.

The purpose of this Symposium was to bring together scientists and engineers from the public and private sectors to discuss their research findings on the health effects of diesel engine emissions and to conclude with a discussion of health risk assessment of diesel exhaust.

The Proceedings are organized into eight main sections corresponding to the format of the Symposium and addressing Physical and Chemical Characteristics of Diesel Emissions, In Vitro Carcinogenic and Mutagenic Effects of Diesel Emissions and Components, Biochemical and Metabolic Effects, Toxicological Effects of Inhaled Diesel Emissions, Mutagenic and Carcinogenic Potency of Extracts of Diesel and Related Environmental Emissions, Mutagenicity of Inhaled Diesel Emissions, Carcinogenic Effects of Exposure to Diesel Emissions, Epidemiological Studies, and lastly a panel discussion on Health Risk Assessment of Diesel Emissions.

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Norman A. Clarke
William Pepelko
Robert Danner

ABSTRACT

The Health Effects Research Laboratory of the U.S. Environmental Protection Agency sponsored an International Symposium on the Health Effects of Diesel Engine Emissions. It was held in Cincinnati, Ohio on December 3-5, 1979.

The Symposium brought together scientists, engineers, and Federal, state, and local public health officials for the purpose of determining the state-of-knowledge regarding the physical and chemical characteristics of diesel emissions, *in vitro* carcinogenic and mutagenic effects of diesel emissions and their components, their biochemical and metabolic effects, the toxicological effects of inhaled diesel emissions, the mutagenic and carcinogenic potency of extracts of diesel and related emissions, the mutagenicity of inhaled diesel emissions, the carcinogenic effects of exposure to diesel emissions, and the results of epidemiological studies involving human exposure to diesel emissions. The Symposium culminated in a panel discussion on the health risk assessment of diesel emissions.

The Proceedings consists of 77 manuscripts and associated discussions, as well as the panel discussion.

ACKNOWLEDGMENTS

The assistance of the many individuals who contributed to the success of this Symposium and the timely completion of the proceedings is gratefully acknowledged. Special appreciation is due to the speakers, for the quality of their presentations and promptness in submitting their papers, the session chairmen and the many participants who contributed to the discussions.

We also wish to acknowledge the assistance of the Center of Environmental Research Information and in particular the efforts of Mr. Larry Dempsey, in providing the many services in connection with arranging the Symposium. We also are indebted to Verna Tilford and Joan Mattox for assisting with registration, and to Deborah Dean, Jean Roe and William B. Peirano who helped in many ways to make the Symposium a success.

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KEYNOTE ADDRESS

Stephen J. Gage
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Washington, DC

I am delighted to be here today addressing this symposium on the health effects from diesel emissions, because that subject is a very good example of the problems associated with environmental regulation based on health effects and how health effects research can and cannot influence the regulatory process. The salient points for discussion are:

1. The effects of concern, principally cancer and mutagenesis in the diesel case, are chornic and slow to develop as opposed to acute.

2. The science behind the effects of concern is not well understood.

3. The effects of concern are expected to be of low probability for an individual, but the exposed population will be large.

4. The effects of concern are currently believed to have a possibility of occurring at all exposure levels no matter how small.

5. The toxic agent is a mixture of literally hundreds of compounds. Furthermore, the mixture varies in ways that are not well understood as a function of such non-precise factors as engine design, operating condition, source of fuel, etc.

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6. The object of possible regulation, in this case the diesel car, is not the only source of the toxic pollutant.

7. The diesel engine is seen by many to offer advantages to society that we need, and need now.

The fact that the effect is slow to develop and chronic, its science not well understood, and its occurrence of low individual probabiity means that whatever health effects research we do will not be related to health effects in human beings in any tightly argued scientific way. First of all, this may mean that epidemiology useful for standard settting probably cannot be done. The expected low probability of the effect means that we probably won't get studies showing a positive result, but on the other hand we won't be able to use the negative results because the power of the studies will not be strong enough for us to say that diesel soot is of no concern, especially when the exposed population is so large. Second, for obvious ethical reasons, exposure of humans cannot be done which leads us to the now familiar arguments of the validity of extrapolating from mouse to man. The subspecies of arguments contained in the mouse to man argument are many and include variation between species in pharmacodynamics, routes of exposure, dose levels, preparation of samples, deposition and clearance, etc. I am, of course, not saying that because of these perturbing factors and lack of good theory and knowledge as to the mechanisms of carcinogenesis, that the animal experiments are essentially useless for assessing human health risk. Such obviously is not the case. I am, however, pointing out that even in the area where health effects research is most applicable, the result is not a scientific answer in the sense that the laws of Newton provide a scientific answer for landing on the moon, but a much vaguer argument, elaborate but still basically post hoc ergo propter hoc. The conclusion that we must draw is that even where science makes its strongest contribution to a regulatory health effects question, when it comes to deciding what regulating to actually do, there remain all the basic arguments about environmental regulation about how much is enough and why. I should note here that the Federal Government's Regulatory Council has generated a cancer policy on how cancer data is to be used for regulatory purposes by the Federal Government.

The use of health effects research gets even less scientific when we with horror discover that for regulation it is not enough to assert that a substance such as diesel soot is a carcinogen for regulatory purposes, but that an estimate of potency is needed as well. This is so because many perceive benefits associated with the generation of such substance and they are not willing to forego them without at least a respectable argument made that the asserted adverse health effects will be in some, and always arguable, sense "significant". The argument is further fueled by EPA's policy (consistent with current science, but not proven by it either) that there is no threshold exposure effect for chemical carcinogen. This assumption means, in the current case, that no matter how small the increased exposure to diesel soot, we will expect some increased number of cancer cases. Obviously, whatever the controversy about going from "mouse to man" for establishing human carcinogenicity, the controversy over a numerical estimate of a substance's potency as a human carcinogen is considerably greater. This is not the place to discuss quantitative cancer risk assessment, but I would like to make on point quite clear. At the present time, quantitative chemical cancer risk assessment at environmental exposures is not science, but an analytical technique consistently using the available health effects data to fulfill a policy and regulatory need for some estimate of a substance's human carcinogenic potency.

The fact that diesel soot is a mixture of hundreds of organic compounds of various types, with the composition of this mixture varying as an unkown function of such imprecise thing as engine design, operating conditions, source of fuel, etc., is another place where the diesel soot regulatory issue is similar to other regulatory problems. While the final word has not been said, some think that the compound by compound identification, potency testing and control design for complex mixtures of chornic toxic substances is not going to be a fruitful approach for regulatory purposes. Assuming arguendo the above we see that a method for estimating carcinogenic potency is needed which 1) can be applied to mixtures, and 2) does not require a long time to get answers. For example, whole animal cancer testing is considered in this sense "too slow". In fact, even when dealing with a pure substance, whole animal cancer tests produce results on a time scale that is felt awkwardly slow by the public and regulators. To put it another way, test procedures are needed to assess relatively quickly whether or not the mixture produced after changes are made in how it is generated, is more or less toxic. To take the present case, if we are to reduce the cancer potency of diesel soot by engine design, short-term tests will be needed if the redesign is not to take forever.

Hence, after pointing out above, the arguments engendered by mouse to man extrapolation, I shall now suggest that research must be aggressively pursued so that we go from microbe or cell to man where feasible. The general problem needing research to develop methods is then short-term tests for chronic effects. The chronic effect of interest with the diesel is cancer, but for general environmental protection the problem is more broad in that such test systems are needed in the areas of chronic lung disease, neurological disorders, immune system impairment, etc.

The Environmental Protection Agency's diesel research effort, as you will hear, is pursuing such research both in the Health Effects Research Laboratory here in Cincinnait and at the one in North Carolina. We currently think that in developing such test systems, three aspects need emphasis. First, the systems should produce very few false positive results, or to say it another way, the false alarm probability should be low. Second, the systems should produce very few false negatives or the detection probably should be high. Third, the systems need to quantitatively assess human potency. I believe I have listed these needed characteristics in order of difficulty, and speaking off the cuff, I think the end is in sight in the case of cancer for a low false alarm (i.e., no or few false positives) short-term test system. At the risk of being slightly parochial, I can think of no area of health effects research that is more crucial to EPA's regulatory needs than the development of such short-term tests for quantitatively and reliably assessing chronic human effects.

Preliminary estimates have indicated that even if diesel cars emitting particles at the current level extensively penetrate the country's automobile fleet that they will not be the only major contributor of diesel particles in the air. As the energy crunch continues, we expect more diesels to show up including such areas as cogeneration of electricity with space heating and cooling, and heavy and light duty trucks. The implications are clear, regulation of one part of a potential diesel problem will affect the regulation of other diesels. This will almost certainly force health effects work on other sources of diesel particles and from there to the health effects of carbon containing particles in the air. Carbon particles (from all sources) are the second most abundant species in the air after sulfates nationwide, and indeed are the most abundant species contributing to fine particles in many western urban locations.

My last point for discussion, namely that many people feel that diesel cars have features that we need and need now, is the one where the contribution of health effects research runs right into all the other things we think are important in life besides health. It is in this area where we health researchers are apt to feel that no one listens to us or if they do, they completely misinterpret what we have to say. All I can say is this is where we as scientists must make the impact of health effects research felt. We must not present our data nad arguments as being more scientific than they are, however, we must not allow ourselves to be pushed aside in our areas of competence. This requires that we educate ourselves so that we understand what regulators think, but furthermore, that we educate the regulators so they understand what we have to say.

In closing, I would like to stress that in spite of the fact that there seems to be no prospect that health effects research will take the controversy out of standard setting or render having to think about a problem obsolete, health effects research is one of the major components in standard setting. It is necessary to keep us from paying an unacceptably high price (in terms of deleterious health effects) for an otherwise beneficient technology or alternatively to keep us from foregoing valuable technology from fear of phantom health effects. This health research must be done and done well. In your pursuit of this work, we all, and indeed the country, must wish you Godspeed. Thank you.

Session I

PHYSICAL AND CHEMICAL CHARACTERISTICS OF DIESEL EMISSIONS

Chairmen:

Dr. Ronald Bradow
Dr. James N. Pitts

Characterization of Diesel Particulate Exposure.
Williams, Ronald L. and David P. Chock.

Characterization of Organic Constituents in Diesel Exhaust Particulates.
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Gibbons, Robert A., and Dr. B. A. Wolf.

CHARACTERIZATION OF DIESEL
PARTICULATE EXPOSURE

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ABSTRACT

This report summarizes our work on the physical and chemical characterization of diesel exhaust with emphasis on the extractable and benzo(a)pyrene content of the particulate. Compared with benzene-ethanol, methylene chloride is an unsuitable extraction solvent for the measurement of benzo(a)pyrene in diesel particulate. Using recently developed analytical techniques, a series of studies of sampling parameters shows that the composition of the particulate is fixed when it leaves the tailpipe and that the composition does not change with dilution. Further, sampling results in a dilution tunnel agree quite well with those observed in open air sampling. Finally, based on various air quality models and projections of the diesel sales-emission scenario, we find that the light-duty diesel will be a minor contributor to total ambient particulate.

INTRODUCTION

In an era constantly threatened by energy shortages, efficient usage of our energy resources is of primary importance. The most attractive feature of diesel engines is clearly their superior fuel economy, as compared to gasoline engines. However, there has been concern expressed in some quarters about the potential health effects of diesel emissions. Extensive research has been conducted for some time at General Motors and elsewhere to assess the potential health effects due to exposure to diesel particulate and other diesel emissions. Fundamental to these efforts is an understanding of the physical and chemical character of diesel emissions, as well as the anticipated levels of human exposure. We will discuss the latest information on the physical and chemical characteristics of diesel emissions and, with that background, assess the air quality impact of expanded usage of light-duty diesel vehicles.

CHARACTERIZATION OF DIESEL EMISSIONS

The facilities used to generate and collect diesel emissions and the details of the chemical analyses will not be described in this paper. Previous publications on these subjects may be consulted (1-3). Instead, we will begin with the analysis of filter samples of diesel particulate and then use those results to examine filter sampling techniques and the potential interactions between the gases and particles emitted by diesel vehicles.

Analysis of Filter Samples

Extraction of Particulate Matter: Solvent extraction is a convenient method for dividing diesel exhaust particulate into two components. The composition of the extraction solvent determines the quantity and composition of the material extracted. We have elected to use a mixture of benzene and ethanol (4:1) in order to extract both the relatively nonpolar organic materials, e.g., hydrocarbons, which are readily soluble in benzene, and the more polar organic materials, e.g., aldehydes, ketones, peroxides, acids, or heterocyclic compounds which tend to be more soluble in ethanol. This choice of solvent was based in part on earlier research in the determination of polycyclic aromatic hydrocarbons in vehicle exhaust (4,5).

Each weighed filter was cut into strips approximately 12 mm x 50 mm and extracted in a 30-mm i.d. Soxhlet apparatus. In order to reduce both the volume of extraction solvent required and the syphon-cycle time, a 19-mm diameter by 80-mm-long glass rod was put into the extraction chamber with the filter. The 125-ml boiling flask with 60-ml extraction solvent and several Teflon boiling-aid chips was set directly into steam to nearly one-half the flask diameter, and the entire assembly was wrapped in a 12-mm-thick glass blanket to reduce heat loss. Syphon cycle-time (the time between batch extractions) was approximately three minutes, and the total heating time was three hours so about 60 syphon-cycles took place. Four extraction units were usually operated simultaneously.

The extract was filtered into a 100-ml beaker through a small, tared glass-fiber filter to remove any carbon particles which may have washed off the sample filter. The extract volume was reduced to a few ml by evaporation of the solvent under a stream of filtered air. The extract concentrate was then transferred to a small, tared vial, and the evaporation of solvent was completed in the same manner. The vial, the extracted exhaust sample-filter, and the extract clearing-filter were stored a minimum of 16 hours in the controlled atmosphere of a balance room and then weighed to determine the weights of extractables and dry soot.

In completing the evaporation of solvent, some of the diesel fuel constituents which would have been extracted will also be evaporated. This loss is minimized by stopping the air flow as soon as the residue appears to be stabilized, i.e., no further reduction in volume or visual absence of solvent (6). Such loss of diesel fuel is probably modest, considering that material balances in the total extraction procedure have averaged above 93%, as shown in Table I.

Table I. Overall Recovery of Particulate Matter in Extraction of Diesel Soot on Filters

Test Series	Number of Filters	Average Percent Accounted For
a	12	94.4 ± 8.7
b	16	98.2 ± 4.8
c	19	99.2 ± 5.5
d	13	93.3 ± 4.7
e	24	96.5 ± 17.4
f	14	96.3 ± 8.8

The percentage of exhaust particulate matter soluble in benzene-ethanol differs among cars, and for individual cars it differs with the mode of operation. In our experience, the soluble fraction has ranged from about 0.1 to 0.9. Only in the case of one car, at low cruise speed, did the soot contain sufficient liquid to soak into the sample filter, giving the filter an oily appearance. The extract was most often a pasty solid, but was sometimes a relatively viscous oil and at other times a dry-appearing solid.

Several other solvents have been used in Soxhlet extractions to determine the extractable mass compared to that obtained with benzene ethanol (Table II). A considerable range in extractable mass was observed. The final choice of an extraction solvent is dictated by the kind of analysis intended for the extracts. These strong organic solvents are well suited for chemical analysis purposes, but they may be inappropriate in biological studies.

Table II. Potential Solvents for Extracting Diesel Particulate

Solvent	Index of Solubility[a]
benzene-ethanol	1.00
methylene chloride	0.66
dichloroethane	0.66
cyclohexane-ethanol	0.93
cyclohexane-isopropanol	0.80
chloroform-ethanol	0.99
dichloroethane-ethanol	1.12
methylene chloride-ethanol	0.88
benzene-isopropanol	0.92
dichloroethane-isopropanol	0.85

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[a] Extractable mass relative to benzene-ethanol

During the course of this work, the EPA recommended that methylene chloride (dichloromethane) be used as the extracting solvent (7). In order to compare the efficiency of methylene chloride with benzene-ethanol for the extraction of benzo(a)pyrene (BaP), a series of extractions were performed using 20-mg portions of diesel exhaust particulate which had been collected in replicate tests. The bulk particulate was wrapped in a fiberglass filter paper and extracted for 3-hours (60 cycles) using either benzene-ethanol (80:20) or methylene chloride. The results are shown in Table III.

Trials 1, 2, and 3 in Table III show that methylene chloride extracts only 60 to 70% as much BaP from diesel exhaust particulate as benzene-ethanol. (Similar experiments using cyclohexane and methanol showed that these

Table III. Comparison of Quantities of BaP Extracted (ng BaP/mg particulate) with Benzene-ethanol and Methylene Chloride

Trial	Extraction	Benzene ethanol	Methylene Chloride
1	3-h Soxhlet	8.93	6.28
2	3-h Soxhlet	9.41	5.50
2A	Second 3-h Soxhlet with fresh solvent	0.75	0.38
3	3-h Soxhlet	7.98	4.94
3A	Second 3-h Soxhlet with the solvents reversed	0.04	3.63

solvents extracted only about 40% as much BaP as benzene-ethanol.) Trial 2A, Table III, shows that a second 3-hr extraction yields only a small amount (7-8%) of BaP. In contrast Trial 3A with the solvents reversed shows that after one 3-hr extraction with methylene chloride considerable benzene ethanol extractable BaP is left on the diesel particulate. This demonstrates convincingly that the low results for methylene chloride extractions are not due to decomposition of BaP in methylene chloride.

To further test the extraction efficiency, several experiments were performed in which the diesel exhaust particulate was "spiked" with a solution of BaP before extraction. These experiments were performed in parallel to those shown in Table III, so a "total expected" BaP value could be calculated from the benzene-ethanol results in Table III plus the spike. These results are shown in Table IV.

The benzene-ethanol results in Table IV show the excellent recovery of the spikes obtained with this solvent. The methylene chloride results show lower recovery and less consistency; the BaP recovery varies from 65 to 86% of the total expected. Finally, Trial 3A again shows that benzene-ethanol effectively extracts additional BaP after a methylene chloride extraction, but the reverse is not true. Since BaP is biologically active and, thus, a reasonable model compound, the extraction results indicate that benzene-ethanol is an efficient extraction solvent but that methylene chloride is totally unsuitable as a solvent for diesel particulate extraction.

Table IV. Recovery of BaP from Spiked Samples of Diesel Exhaust Particulate (ng BaP/mg particulate) Extracted with Benzene-ethanol and Methylene Chloride

		Benzene-ethanol		Methylene Chloride	
Trial	Description	Total Expected	Found	Total Expected	Found
1	3-h Soxhlet	13.2	13.9	12.5	8.16
2	3-h Soxhlet	15.1	14.8	16.6	14.3
3	3-h Soxhlet	14.8	14.2	14.8	12.1
3A	Second 3-h Soxhlet with the solvents reversed	--	0.08	--	3.05

Analyses for Carbon, Hydrogen, Oxygen, and Nitrogen: Elemental analysis of extracts and of the dry soot remaining after the extraction has been obtained for carbon, hydrogen, oxygen, and nitrogen (C, H, O, N). The methods employ combustion oxidation/reduction according to Pregl or Dumas, and utilize a Perkin-Elmer model-240 elemental analyzer. Typical results are illustrated for a series of seven extracted filters and the extracts of two of those filters (Table V). The extract and the dry soot remaining after extraction both contain considerable oxygen. The principal difference in composition is the lower hydrogen and the higher carbon content of the dry soot.

Table V. Elemental Analyses of Diesel Exhaust Particulate Matter

Benzene-ethanol Extract

Vehicle	Weight %			
Operation	C	H	O	N
48 km/h	77.1	10.9	11.3	0.70
Idle	72.8	7.4	19.0	0.73

Dry Soot (material remaining after extraction with benzene-ethanol)

Vehicle	Weight %			
Operation	C	H	O	N
48 km/h	81.9	1.4	16.2	0.60
64	78.8	1.6	18.9	0.70
80	84.7	1.6	12.9	0.73
96	83.6	3.6	11.4	1.40
S-7	82.6	2.7	14.1	0.59
HFE	76.5	2.4	20.3	0.90
Idle	75.3	1.7	22.1	0.96

Molecular Weight Distribution by Gel Permeation Chromatography (GPC): Extracts of soot from several cars were analyzed for molecular weight distribution by gel permeation chromatography, also sometimes referred to as liquid exclusion chromatography. The gel column was one commonly used for styrene polymers and therefore was optimized for relatively large molecules. Relatively low concentrations of substances below a molecular weight of about 150 could not be measured accurately. A typical GPC chromatogram for a diesel particulate extract is illustrated in Fig. 1a, along with chromatograms for diesel fuel, Fig. 1b, and SAE-30 lubricating oil, Fig. 1c. It is evident that the molecular-weight-distribution curve for the soot extract is more nearly like the distribution for the lubricating oil than the distribution for the diesel fuel. The extract is shown to include more components with high molecular weights than lubricating oil, but, because the extract contains more polar oxidation products, the actual molecular weight distribution may differ from the calibrations with polystyrene. Because the GPC procedure doesn't apply thermal stress to the sample, it is more appropriate than distillation or the GC-simulated distillation procedure for the determination of molecular weights above about 600.

A summary of average molecular weights for several extracts is given in Table VI. M_N refers to the number-average molecular weight and M_W to the weight-average molecular weight. For the purpose of calculating a mean-molecular formula, M_N is utilized to show the mean-molecular size by frequency of occurrence.

Table VI. Molecular Weight of Extracts of Typical Exhaust Particulate Matter Compared to Diesel Fuel and Lubricating Oil

Operating Condition	M_N	M_W	Range
48 cruise	369	476	150-5000
64 cruise	381	552	150-5000
80 cruise	394	647	200-7600
96 cruise	371	619	200-7600
S-7 cycle	400	607	150-5000
HFE cycle	385	520	150-5000
Idle	384	531	150-5000
Diesel Fuel	199	223	150-550
SAE #30 Lubricating Oil			
Fresh	443	584	200-3000
Used	487	595	200-3000

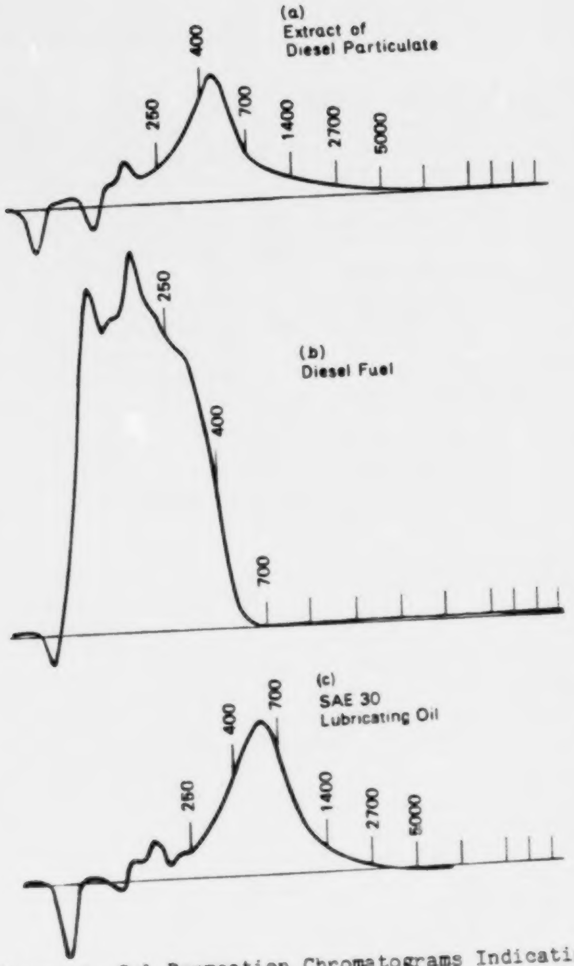

Figure 1. Gel Permeation Chromatograms Indicating Molecular Weight Distribution Based on Polystyrene.

Utilizing M_N values from Table VI and the elemental compositions from Table V, the general molecular formulae for the extract samples would be:

30 mph $C_{24} H_{39} O_{2.6} N_{0.18}$
Idle $C_{23} H_{29} O_{4.7} N_{0.21}$

The mean molecular weight M_N of the extract under all operating conditions is somewhat lower than that of SAE-30 lubricating oil ($M_N = C_{31} H_{58}$), but the distribution covers a wider range.

Infrared Absorption Spectroscopy: Infrared absorption spectroscopy has been utilized to obtain structural information on the benzene-ethanol extracts of diesel particulate. The method, as applied, is semiquantitative. Because of the viscous nature of the samples and the small sample quantities available, the spectra were obtained by the attenuated total reflectance (ATR) technique. For most extracts, carbon-hydrogen structures similar to those in lubricating oil predominated. Aromatic absorption at 6.25 microns was also similar to that in lubricating oil and diesel fuel. In addition, strong absorptions in the 5.85 micrometer region, indicative of C=O structure, and in the 2.8-3.1 micrometer region, indicative of OH or NH structures, were present. Thus, the extracts contained a substantial fraction of partially oxidized hydrocarbons, e.g., ketones, aldehydes, or acids. The relatively strong C=O and OH structures were supported by the oxygen content, as determined by elemental analysis.

Volatility by Thermogravimetry: The analytical technique of thermogravimetry (TGA) determines the change in sample mass as a function of temperature. Although the technique can be as simple as periodically weighing a sample at room temperature or other controlled temperature (static mode), it often finds its most useful application in the dynamic mode by recording the sample mass as a function of continuously increasing temperature. The sample, in close proximity to a small thermocouple, is suspended on a sensitive balance in a small furnace through which gas of controlled composition flows. For all samples analyzed by TGA, the temperature rise was 20°C per minute to 700°C, starting at room temperature (about 23°C). Gas flow was 100 ml per minute. Samples were heated first in nitrogen to 500°C, then in air to 700°C.

This approach distinguishes between volatile material and combustible material, as shown in Fig. 2. Using the thermogram, one can replot the mass removed relative to the total mass removed at 700°C in air. Results for diesel particulate are illustrated in Fig. 3, along with results for diesel fuel and SAE-30 lubricating oil. It is evident that the particulate matter contained components which overlap the high boiling end of diesel fuel. However, the majority of the weight loss was associated with material less volatile than diesel fuel, i.e., mainly in the volatility range of lubricating oil.

The weight loss (in air) above about 450°C in the TGA procedure is due mainly to combustion of carbon, but also

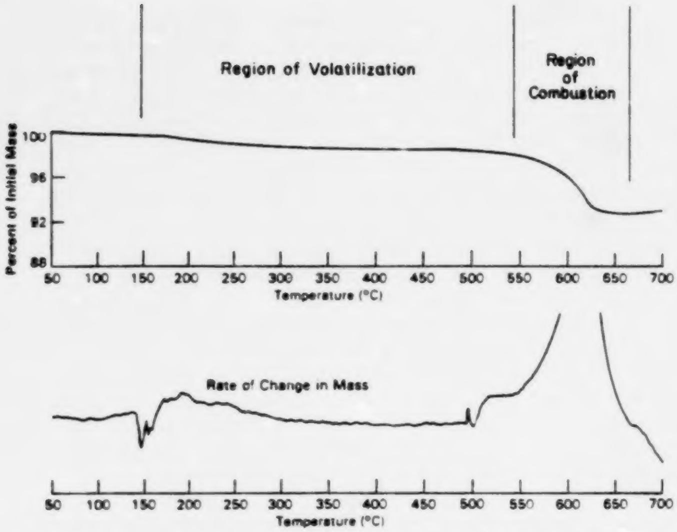

Figure 2. Typical Thermogram of Diesel Exhaust Particulate (On Glass Fiber Filter) Heated to 500°C in Nitrogen, Then Reheated to 700°C in Air.

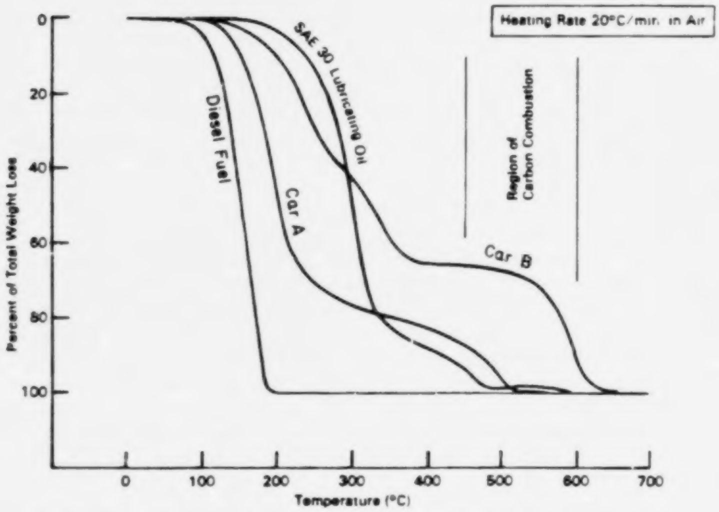

Figure 3. Temperature Dependence of Weight Loss of Diesel Exhaust Particulate (calculated percent weight loss based on combustion end point).

to the combustion of a smaller amount of high-molecular-weight organic matter, the ignition of which causes the composite to burn at a lower temperature. For example, if a particulate sample (on glass-fiber filter paper) is heated in air, the onset of the carbon combustion is in the region of 475-525°C. If, however, the sample is first heated in nitrogen to 500°C, the temperature for the onset of the carbon combustion in air is 600-625°C, an elevation of about 100°C.

The TGA process is not a vapor/liquid equilibrium process. The temperature in the TGA apparatus at which 50% of the diesel fuel is lost (Fig. 3) is 150°C. At this temperature, the equilibrium vapor pressure of the midpoint fraction of diesel fuel is about 10 torr (8). Rapid loss of diesel fuel in the TGA apparatus occurs about 100°C below the normal boiling point of the midpoint fraction of diesel fuel. For lubricating oil, the midpoint in the TGA procedure (Fig. 3) is about 300°C, at which temperature the vapor pressure of the midpoint fraction is also about 10 torr. In this case, the TGA removal temperature is about 180°C below the normal boiling point of the midpoint fraction.

Correlation of Extractables with Volatiles: Solvent extraction separates the total particulate into two fractions which can be used in chemical and biological analysis. Thermogravimetry may be used to determine the mass of volatile material and the mass of combustible material in a particulate sample, but it destroys the sample. However, the relative simplicity of thermogravimetry makes it an attractive measurement tool if it yields results similar to extraction results. Depending on the diesel engine involved, we have found various levels of correlation between these two methods. The results for one particular diesel vehicle are shown in Fig. 4. In this example the solvent extractables and TGA volatiles agree very well. The least-squares best fit through the origin has a slope of 1.04, and the correlation coefficient is 0.982. A wide range of driving modes are included in the data set and the extractables cover nearly the full range of extractables observed for any diesel. Using other diesel cars, unexplained deviations from this simple correlation have been observed, as shown in Fig. 5. Thermogravimetry remains an attractive alternative to solvent extraction but care must be exercised in its application and interpretation.

Filter Sampling Techniques

Verification of the Filter Sampling Technique: Although filter sampling is commonly used to collect samples of particulate emissions from a variety of sources

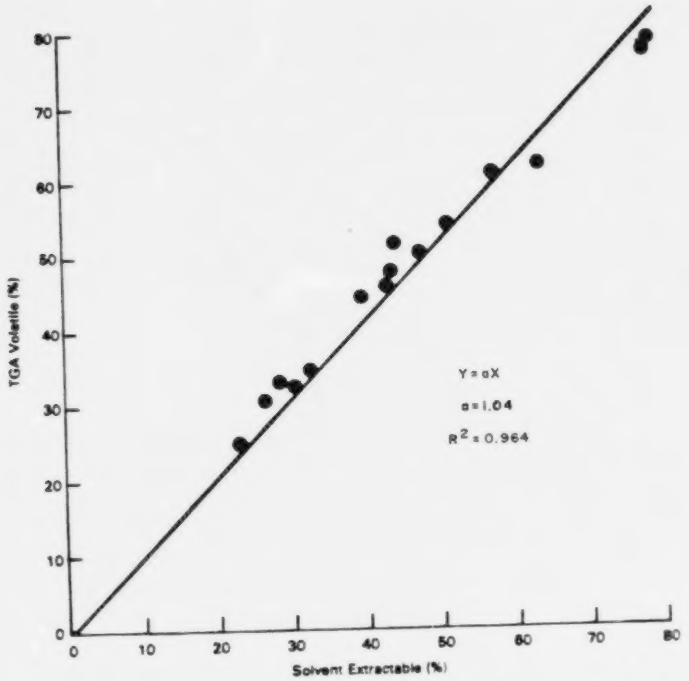

Figure 4. Correlation of TGA Volatiles with Solvent Extractables for Cyclic and Cruise Driving of One Diesel Car.

(9,10), very little is known about changes in the particles which might occur during the collection process, particularly when the source is a diesel-powered automobile. This part of our report describes a series of experiments which were designed to examine some of the factors which might cause changes in diesel particulate during the collection and analysis of the filter samples. These experiments include: 1) sampling rate variations, 2) sampling time variations, 3) additional exposure to diesel exhaust gases, and 4) stability during filter-sample storage.

a) Sampling Rate Variations: Sampling rate is one of the factors which could change the quantity and composition of diesel particulate collected on a filter. A set of nine filters was collected from the dilution tunnel at sampling rates which ranged from 0.062 to 0.276 m^3/min, each with a sampling period of 10.0 min. The concentration of particulate in the sample stream was calculated from the mass on the filter and the volume of diluted exhaust sampled.

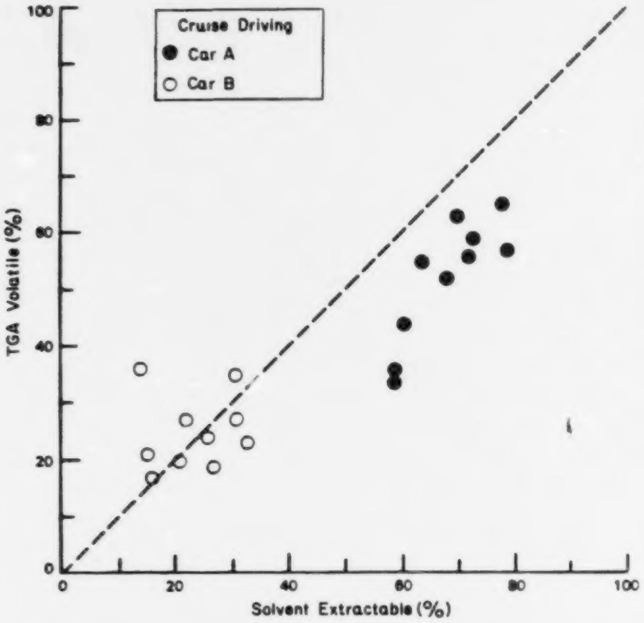

Figure 5. Weak Correlation Between TGA Volatiles and Solvent Extractables for Two Diesel Vehicles in Cruise Driving.

The extractable percentage (benzene-ethanol solvent) and the BaP concentration in the particulate were also determined. The results of the sampling rate experiment are shown in Table VII.

If filter efficiency is low, we would expect the efficiency to increase with loading of particles on the filter. However, for a filter with high efficiency, the loading rate on the filter will be proportional to the sampling rate. For each filter in this study, the mass concentration of particulate in the stream sampled was calculated (see Table VII). The mass concentration was 9.89 ± 0.16 mg/m^3, and there was no discernible trend with sampling rate, which confirms the high efficiency of the Dexiglass filters.

For the range of sampling rates studied, the extractable percentage was 36.2 ± 1.8, and the BaP content was 10.2 ± 1.1 ng/mg. Neither the extractable percentage, which would reflect adsorption, nor the BaP concentration, which would reflect chemical reactions, showed any dependence on sampling rate.

Table VII. Effect of Sampling Rate on Diesel Exhaust Particulate

Sample Number	Sampling Rate[a] (m^3/min)	Particulate Mass Concentration (mg/m^3)	Extractable Percentage	BaP Concentration (ng/mg)
1	0.062	9.91	32.8	8.0
2	0.092	9.70	38.9	9.4
3	0.120	9.81	37.9	11.3
4	0.149	9.76	35.6	9.8
5	0.175	9.81	36.6	9.9
6	0.200	10.09	36.7	9.8
7	0.229	10.17	36.9	11.4
8	0.252	9.93	34.4	10.8
9	0.276	9.79	36.2	11.2
		9.89 ± 0.16[b]	36.2 ± 1.8	10.2 ± 1.1

---

[a] Sampling period 10.0 min.

[b] One standard deviation.

b) Sampling Time Variations: A set of eight filter samples was collected from the dilution tunnel at a constant sampling rate of 0.20 m^3/min, but the sampling period was varied from 1 to 25 minutes. The concentration of particulate in the sample stream, the extractable percentage, and the BaP concentration are shown in Table VIII. The range of sampling times covers the practical range commonly used in vehicle testing.

Any change in collection efficiency which might occur during extended sampling periods would lead to changes in the apparent mass concentration in the diluted exhaust. The mass concentration was 9.46 ± 0.35 mg/m^3, with no dependence on the duration of the sampling period. Processes such as adsorption and chemical reaction could affect the composition of the particulate on the filter since particles collected in the initial layer of particulate are exposed to gaseous emission components transmitted by particle layers collected later. As can be seen in Table VIII, the extractable percentage and the BaP concentration were again constant with no dependence on the duration of the sampling period.

Therefore, variations in flow rate and in the duration of the sampling period have no effect on the particle mass concentration, the extractable percentage, or the BaP concentration. Over the practical range of these two variables we find no evidence for filter efficiency changes, adsorption, or chemical reaction.

c) Additional Exposure to Diesel Exhaust Gases: A set of four identical filter samples were collected from the dilution tunnel to study the effect of additional exposure of particulate to diesel exhaust gases. The sampling period for these filters was 10 minutes during which about 20 mg of particulate were collected. To complete the experiment, two filter holders were connected in series for additional sampling. A clean filter was placed in the first holder to remove the particulate. Two of the previously loaded filters were placed one at a time in the second holder for exposure to diluted exhaust gas. One was exposed for one minute, and a second filter was exposed for 10 minutes. The extractable percentage and the BaP concentration in the particulate were determined on the four filters (Table IX, Set 1). This experiment was repeated using lighter loaded filters, collected under different driving conditions (Table IX, Set 2).

In both sets the additional exposure to diesel exhaust gases caused no significant change in mass on the filter, in the extractable percentage, or in the BaP concentration. Again, it appears that interactions between the

Table VIII. Effect of Sampling Period on Diesel Exhaust Particulate

Sample Number	Sampling Period[a] (min)	Particulate Mass Concentration (mg/m^3)	Extractable Percentage	BaP Concentration (ng/mg)
1	1	10.03	43.0	7.5
2	3	9.77	43.4	9.9
3	5	9.76	38.5	10.3
4	7	9.23	37.7	10.6
5	10	9.14	37.5	9.3
6	15	9.07	40.1	10.4
7	20	9.43	40.7	9.1
8	25	9.27	38.5	9.2
		9.46 ± 0.35[b]	39.9 ± 2.3	9.5 ± 1.0

---

[a] Sampling rate, 0.20 m^3/min

[b] One standard deviation.

Table IX. Effect of Additional Exposure to Diesel Exhaust Gases

Sample Number		Total Particulate (mg)	Additional Exposure (min)	Extractable Percentage	BaP Concentration (ng/mg)
Set 1	1	19.88	0	33.3	8.9
	2	19.83	0	33.0	8.2
	3	20.01	1	32.7	8.6
	4	20.39	10	30.6	8.8
Set 2	5	7.53	0	30.2	4.9
	6	7.43	1	31.9	5.2
	7	7.52	10	31.3	5.4

diesel exhaust gases and particles in this sampling system have reached a steady state before the particles are collected on the Dexiglass filter.

d) Stability During Filter Sample Storage: Eleven additional replicate filter samples were collected from the dilution tunnel for a filter storage study. The amount of particulate collected on each filter was 7.5 ± 0.2 mg. These filter samples were stored in a room at 24 ± 0.5°C and 45 ± 1% relative humidity and with normal fluorescent light. Periodically, up to 150 days after collection, filters were randomly selected for analysis. The extractable percentage and the BaP concentration are listed in Table X.

Table X. Effect of Filter Sample Storage on Diesel Exhaust Particulate

Sample Number	Storage Period (days)	Extractable Percentage	BaP Concentration (ng/mg)
1	9	34.1	12.1
3	9	32.9	11.1
3	9	32.2	11.1
4	9	32.0	10.9
5	28	38.8	9.8
6	28	39.2	9.0
7	28	40.0	10.1
8	91	44.6	7.7
9	91	40.1	6.3
10	147	36.5	5.5
11	147	37.8	4.3
		37.1 ± 4.0[a]	

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[a] One standard deviation.

While the extractable percentage has a standard deviation of 4.0%, it shows no systematic change with storage time. The total BaP loss in 150 days was 57% and the BaP concentration decreased linearly with time at a rate of 0.046 ng/mg/day. While long term storage does cause a decrease in the BaP concentration, the loss in 20 days of storage is less than one standard deviation normally found for BaP in replicate samples analyzed on the same day. To avoid excessive loss of BaP by unidentified processes, the storage conditions should be carefully controlled, and good practice would dictate minimizing storage time.

Comparison of Dilution Tunnel Sampling with Open Air Sampling: When diesel exhaust exits the tailpipe, it is diluted very rapidly. Roadway experiments have shown that exhaust is diluted at least 1000-fold in the first fraction of a second after it enters the free atmosphere (11). This, of course, results in a very rapid decrease in both the temperature and the concentration of the exhaust constituents. In order to collect samples of sufficient size without the confounding influence of large quantities of extraneous ambient particulate and without requiring prohibitively large sampling equipment, certain compromises must be made in the test procedure. In general, vehicle sampling has been carried out in dilution tunnels at five- to twenty-fold dilution.

The question, of course, is to determine what actually happens in the atmosphere. To this end, we have carried out a series of experiments to make a direct comparison between dilution in a tunnel and dilution in the free atmosphere. A vehicle was allowed to idle in a partially enclosed courtyard area and samples were taken at different distances from the tailpipe to provide various dilution ratios. At the same time, CO_2 measurements were taken at the sampling point to provide a measure of the dilution. The range of dilution in these experiments was 2 to 350. The mass of particulate relative to CO_2 concentration was independent of the dilution ratio, as shown in Table XI. Two measures of particulate composition were examined: the extractable fraction and the BaP content. The same vehicle was then tested using a dilution tunnel at two pump speeds which correspond to dilution ratios of 14 to 1 and 26 to 1. These data points are also included in Table XI, and it can be seen that the two experiments agree within experimental error. The extractable fraction does not change with dilution. The fact that the BaP content tends to increase with dilution is interesting. Our interpretation of this data is that sampling in a concentrated exhaust stream causes significant BaP to be converted to other products, probably by reaction with NO_2. These results may have important implications in the area of biological testing of diesel particulate material. These experiments strongly suggest that the hydrocarbon is bound to the particles as they leave the tailpipe and that the hydrocarbon remains attached, at least through the initial dilution process.

Table XI. The Effect of Dilution Ratio on Particulate Mass Concentration and Composition

	Dilution Ratio	No. of Samples	Specific Particulate Concentration ($\mu g/m^3$ particulate/ppm of CO_2)	Extractables (%)	BaP (ng/mg)
Open Air	< 2	1	--[a]	15	5
	5.1-7.0	5	5.0	26	19
	11-17	4	4.1	28	22
	21-24	3	3.5	28	28
	44-88	4	4.5	29	25
	135-150	2	6.5	28	32
	220-350	2	3.7	--[b]	20[c]
Dilution Tunnel	13.8	1	3.9	27	23
	26.5	1	3.8	27	22

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[a] CO_2 concentration was above instrument capability.

[b] Extractable mass was too small for accurate determination.

[c] Large background correction to mass makes this number uncertain.

Filter sampling is intended to separate an aerosol into its gaseous and particulate components. Concern has been expressed about the potential desorption of hydrocarbons from diesel particles and the potential for such gases to participate in atmospheric photochemical reactions. To begin answering these questions, we have carried out a series of studies in which the weights of filters containing diesel particulate were monitored for as long as seven months (Table XII). The average weight loss of the particulate on the filters was only 0.40%. The extractable portion of the particulate in this set of samples ranged from 15% to 45%, representative of the wide range of sampling and operating conditions used to generate these samples. By way of contrast, an experiment in which diesel fuel was added to a clean filter resulted in 94% evaporation of the fuel in 40 days. The lack of desorption of hydrocarbons from diesel particulate is certainly not surprising since it is well known that the vapor pressure of hydrocarbons is dramatically reduced when they are adsorbed on carbon (12,13).

Table XII. Evaporation of HC from Diesel Particulate Aged on Filters (room temperature storage)

Days After Collection	Particulate Mass on Filter (mg)	% of Particulate Mass Evaporated	% of Particulate Mass Extractable*
80	17.7	0.29	28
80	19.5	2.05	37
80	18.6	0.84	36
80	39.2	0.02	16
80	32.4	0.19	17
190	52.8	-0.10	15
190	38.7	-0.07	17
190	37.4	-0.05	22
190	42.6	-0.41	18
190	29.1	0.97	41
210	16.8	0.35	29
210	17.5	0.10	30
210	34.8	0.80	42
210	34.5	0.58	45
	Avg. % Evaporated	0.40	

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* Determined 24 hours after sample collection on paired filter.

When considering even longer periods of time, it is important to remember that the control of exhaust hydrocarbon emissions is directed toward the reduction of ozone (formed by a photochemical process). The hydrocarbons attached to diesel particulates tend to have very high molecular weights (14), i.e., almost all the hydrocarbons are larger then C_{12}. High-molecular-weight hydrocarbons such as these tend to be very unreactive, and when they react the products tend to be more polar and even less volatile. These considerations suggest that the heavy hydrocarbons are deposited on diesel particulates before they enter the atmosphere and that their reactivity is so low that they will make a negligible impact on photochemical ozone formation.

- Gaseous Emissions

While the issue of potential health effects of diesel engine emissions centers on particulate emissions, chemical characterization and biological effects studies should not ignore gaseous emissions which occur concurrently with the particulate. A list of exhaust emissions from an experimental 5.7 L diesel under Federal Test Procedure conditions is presented in Table XIII. The raw (undiluted) exhaust concentration of each component was calculated using an exhaust flow rate estimated from the average tunnel dilution ratio. The total particulate concentration in the raw exhaust was 85 mg/m^3, from which the other concentrations may be scaled in animal exposure studies using diluted diesel exhaust. Similarly, any interactions which might occur between the gases and particles emitted from diesels must occur at these same relative concentrations regardless of the degree of dilution. Inadequate dilution of the gaseous emissions might give rise to effects in extended animal exposures regardless of the character of the particulate under study.

AIR QUALITY IMPACT

Emission Standards and Diesel Market Share

The Proposed Standards: While the health effects of diesel particulate have been under intense study for some time, to date, no adverse health effects have been observed. The extent of any diesel health effects will surely depend on the level of exposure. In order to limit exposure, the Environmental Protection Agency (EPA) proposed on February 1, 1979 a particulate emission standard for light-duty diesel vehicles: 0.38 g/km (0.6 g/mile) for the 1981 and 1982 model years, 0.12 g/km (0.2 g/mile) for the 1983 model year and thereafter (15).

Table XIII. Exhaust Emissions from an Experimental Diesel (FTP data)

Emission	Mass Emission Rate	Conc. in Raw Exhaust[a]
NO_x	0.77 g/km	82 ppm
NO_2	0.14 "	15 ppm
HC	0.24 "	27 ppm
CO	1.06 "	186 ppm
Sulfur Dioxide	515 mg/km	40 ppm
Total Aldehydes	12 "	1.3 ppm
Hydrogen Cyanide	1.2 "	0.2 ppm
Ammonia	0.6 "	0.2 ppm
Sulfate	8.0 "	1.8 mg/m^3
Total Particulate	380 "	85 mg/m^3
Benzo(a)pyrene	1.9 µg/km	0.43 µg/m^3

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[a] Calculated using exhaust flow rate estimated from average tunnel dilution ratio.

According to the accompanying EPA documents, this emission standard was based on the total suspended particulate air quality standards and on EPA's judgement of technological feasibility. From both the technology and the air quality viewpoints, the proposed standard appears to be very stringent, particularly in the initial period. In response to EPA's proposal, General Motors proposed on April 19, 1979 a Corporate Average Particulate Standard (CAPS) which represents an average over the entire fleet of light-duty vehicles produced by a manufacturer in one model year (16). In this proposal, an eventual corporate average particulate standard of 0.031 g/km (0.05 g/mile) was suggested for the 1987 model year and thereafter. If the market share of light-duty diesels is expected to stabilize at about 25% this standard represents an average emission rate of 0.12 g/km (0.2 g/mile) for the diesel fleet, which agrees with EPA's proposed diesel particulate emission standard for the model year 1983 and thereafter.

Diesel Penetration in the Light-Duty Fleet: In the PEDCo report (17), on which EPA's proposed standard was based, two diesel sales scenarios were considered: the "best estimate" which assumed an ultimate market share of 10%, reached by model year 1983; and the "maximum estimate" which assumed an ultimate market share of 25%, reached by model year 1983. Our estimate, on the other hand, assumed that an ultimate market share of 25% would not be reached until model year 1990. For the purpose of estimating the air quality impact, we assumed that the diesel portion of the total vehicle-miles traveled by light-duty vehicles would be 25%. This latter assumption requires the maintenance of a market share of 25% for about 10 years or more. Based on GM's and EPA's ("maximum") diesel sales projections, the above assumptions may be realized by the year 2000, and in any case the total particulate from light-duty diesels prior to 2000 would not exceed the rate for the year 2000.

Projected Particulate Concentrations due to Light-Duty Diesels

Concentration Estimates: The primary National Ambient Air Quality Standards for total suspended particulate are 75 $\mu g/m^3$ (annual average) and 260 $\mu g/m^3$ (24-hour average). Physically, time averaging has the same effects as spatial averaging. The extent of spatial averaging depends on the emission-source distribution and the fluctuations of the wind. For an urban area, because of the rather diffuse distribution of motor vehicles, the roadside annual average concentration due to emissions from motor vehicles should be essentially equivalent to the regional average over the whole urban area. An upper bound for this number would be the average observed at or near the downtown of an urban area. The 24-hour average, on the other hand, represents a local average for, say, a portion of a downtown area. From observations (18,19), the maximum 24-hour averages are about 2.5 times higher than the annual averages. In the existing total-suspended-particulate air quality standard, however, the ratio of the 24-hour average to the annual average is about 3.5 which means that the annual average is a more stringent standard.

In this connection, it should be mentioned that in the PEDCo study (17), in addition to the regional annual (geometric) mean, a roadside annual (geometric) mean was also defined. (There is no compelling reason to favor a geometric mean or an arithmetic mean. The geometric mean derives from the assumption that concentrations averaged over a fixed duration are lognormally distributed if each of these concentrations are treated as an independent statistical variate. While lognormality is not always appropriate, it is not a major concern here since, in general, the two means are not very far apart -- typically within 10% of each other. It can be proven that for positive quantities, such as concentrations, the arithmetic mean is greater than or equal to the geometric mean.) The roadside annual mean should be essentially the same as the regional annual mean, especially when the upper bounds are considered. Unfortunately, PEDCo arbitrarily multiplied the regional mean by a factor of 11 to obtain the roadside mean. The factor of 11 defies common sense. Worse yet, PEDCo also estimated a regional 24-hour maximum which was assumed to be about three times the regional annual mean. This regional 24-hour maximum was then raised by a factor of 11 to obtain a roadside 24-hour maximum. In other words, the roadside 24-hour maximum is now about 33 times the regional annual mean, whereas observations indicate that the factor is typically 2.5.

To show how much EPA and PEDCo overestimated the roadside concentration, we will compare their estimate with one of our own. We use the worst-case meteorological condition (stable, parallel wind at less than 1 m/s) observed during the General Motors Sulfate Dispersion Experiment (11) and by scaling the observed data, estimate the concentration at 3.5 meters above ground and 3.8 meters from the road. The concentration is 9.2 $\mu g/m^3$ for a traffic density of 17000 cars per day, with 25% of the cars being light-duty diesels emitting 0.6 g/km (1 g/mile) of particulate matter. Since the worst-case condition cannot be sustained throughout the day, we estimate the highest 24-hour concentration to be about one-half of 9.2 $\mu g/m^3$, or 4.6 $\mu g/m^3$. EPA, on the other hand, estimated a value of 52.9 $\mu g/m^3$, at a position three meters above ground and 4 meters from the road. This is about 11 times too high! (The 52.9 $\mu g/m^3$ estimate is the light-duty diesel portion of the total mobile-source estimate of 75.6 $\mu g/m^3$. See EPA (20), Table IV-5.)

There are many existing models for describing short-term (up to 24-hour) and long-term (annual) averages. In particular, the tracer (or surrogate) model is very simple to use. It relates the unknown concentration with the observed concentration of a tracer (e.g., lead), by multiplying the latter by a scaling factor which is the ratio of diesel particulate and tracer emission rates. It is quite reliable for long-term estimates, but is probably less useful for short-term estimates because short-term averages are more sensitive to local fluctuations. Yet, comparisons of the tracer model estimates are consistent with those from the Simple Line-Source Model (21) for worst-case short-term concentrations. Therefore, the tracer model offers a reliable methodology for both long-term-regional and short-term-maximum concentration estimates. In what follows, the tracer model will be used in our concentration estimation. We shall assume a growth rate of 1% per year for the total vehicle miles traveled, a stabilized fleet of 25% light-duty diesels by the year 2000, and a particulate emission of 0.12 g/km (0.2 g/mile). The estimated contributions from light-duty diesels to particulate concentration in 2000 for selected urban areas are shown in Table XIV. It can be seen that the contributions from light-duty diesels are a small fraction of the air quality standard for total suspended particulate. It should also be pointed out that the estimated concentrations are for worst cases in the downtown areas where particulate emissions from mobile sources are emphasized.

Table XIV. Projected Particulate Concentration Due to Light-Duty Diesels for Various Cities in the Year 2000 (μg/m^3)

	Regional Annual Mean	Roadside 24-Hour Maximum
Los Angeles Freeways	10.5	26.2
Los Angeles Downtown	6.0	15.0
Chicago, New York Downtown	3.5	8.8
Cincinnati, Denver, Philadelphia Downtown	2.0	5.0

Manhattan Taxi Cab Scenario: In the foregoing we implicitly assumed that light-duty diesels are evenly distributed throughout the country. It has been suggested that this might not be the case. For example, all taxis in Manhattan, New York might be powered by diesels. According to the New York City Traffic Bureau (22), the traffic density on major streets is about 400 vehicles per hour per lane during a typical rush-hour period, with 50% taxis, 26% passenger cars, 20% trucks, and 4% buses. We assumed that 60% of the vehicles were light-duty diesels. These should cover the entire taxi fleet and a substantial portion of the private-passenger cars. We also chose a high emission rate of 0.4 g/km (0.7 g/mile) for all light-duty diesels. Then, using the APRAC Street Canyon Model (23) under the worst meteorological conditions, we estimated the light-duty diesels contribution to the roadside 24-hour maximum concentration to be 40 μg/m^3. This concentration should be compared to the 24-hour particulate air quality standard of 260 μg/m^3.

DISCUSSION

A detailed study of solvents, using BaP as a model compound, showed that benzene-ethanol is an efficient solvent for extracting BaP from diesel particulate while methylene chloride is unsuitable because it gives lower and less consistent BaP recoveries. Various chemical and physical analyses showed that diesel particulate extract resembles slightly oxidized engine lubricating oil.

A series of studies of sampling parameters showed that Dexiglass filters are highly efficient filters for diesel particulate, and that the composition of the particulate is fixed when it leaves the tailpipe. As a result, the composition is relatively stable in diluted exhaust. The particulate composition is also quite stable to storage, but a slow degradation of BaP (50% in 150 days) was observed. Sampling results in a dilution tunnel agreed quite well with those observed in open air sampling.

The concentrations of some of the gaseous emissions which accompany the particulate emissions from diesels were presented. Inadequate dilution of these gaseous emissions, which are similar to those from gasoline engines, might give rise to effects in extended animal exposures regardless of the character of the particulate under study.

Based on various air quality models and on our projections of the diesel sales-emission scenario, it is estimated that the light-duty diesel will be a minor contributor to ambient particulate concentrations. These diesel emissions will maximize in the year 2000 at about 2 $\mu g/m^3$ (annual average) for most cities and at less than 10 $\mu g/m^3$ (annual average) in the worst locations. Roadside 24-hour maximum concentrations will be typically 2.5 times higher than the regional annual means, in contrast to EPA's earlier estimation of a factor of 33.

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General Discussion

B. PETERS: Since there is some concern that a lot of the potency could be due to oxygenated polycyclics, do you have any information on other pure polycyclic compounds besides BaP?

R. WILLIAMS: We have a limited amount of information on other pure polycyclics. We have no analytical information on the oxygenated polycyclics derived from the pure parent compounds.

R. FREEDMAN: Have you tried toluene-ethanol, and if so, how does it compare to benzene-ethanol?

R. WILLIAMS: Yes, we have tried toluene-ethanol. We do find comparable results. I think the important ingredient here is the combination of an aromatic solvent and an alcohol when one is comparing mass, and also perhaps for the penetration of particles for the extraction of tightly bound materials like BaP. I would say we find very comparable results, mass-wide and BaP-wise for toluene-ethanol compared to benzene-ethanol. Since I am also involved with some of the CRC Chemical Characterization Panel's work, we worry a great deal about benzene. We need to lock at solvents other than benzene, and I think toluene would be a very, very good substitute material.

A. LAWSON: You said the gas particle conversion of the tailpipe is status stay. What does that amount to in terms of dilution ratios in your tunnel?

R. WILLIAMS: The dilution ratios covered in the open air samples range from five to 350. Our tunnel can be run at a dilution ratio of either 14 or 26. We have numerous samples in that low dilution ratio range where the mass of material is easily gathered. Significantly less material was collected at 300 to one, but the data shown was for the entire range. In the paper you will see the numerical data for all of the samples we collected.

A. LAWSON: Are you saying that in the tunnel, if you get 14 to one dilution you will be reaching status stay?

R. WILLIAMS: A dilution ratio of 14, and even five to one, is in good agreement with that value.

CHARACTERIZATION OF ORGANIC CONSTITUENTS IN DIESEL EXHAUST PARTICULATES

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ABSTRACT

The diesel engine is experiencing increased use outside the industrial sector, particularly in the area of automotive propulsion. Concern has arisen over the possibly toxic organic compounds released to the atmosphere as constituents of the large amounts of particulate matter present in diesel exhaust. The objective of the program reported here was the preparation of large amounts of organics extracted from diesel exhaust particulates for submission to biotoxicity testing. Large quantities of particulates collected on glass fiber filters were extracted in a Soxhlet apparatus and fractionated into seven groups of compounds. The fractions were submitted for toxicity testing by various in-vitro methods which then guided subsequent analytical chemical characterizations. Analytical determinations included elemental analysis, infrared spectroscopy, flame photometric gas chromatography, and gas chromatography/mass spectrometry. The work was of a preliminary nature and resulted in the tentative identification of approximately 40 compounds in three organic fractions.

The diesel engine has been shown to be more fuel efficient than most other types of propulsive power plants and it produces lower emissions of some of the gaseous pollutants. However, there is still concern about potentially toxic pollutants in the particulate emissions which form a significant fraction of diesel exhaust emissions. The solids are known to contain toxic organic compounds some of which are carcinogenic, for example benzo(a)pyrene.

Consequently, a great deal of work has been done at many institutions in attempts to characterize the production and effects of this compound and others like it.

The Department of Automotive Emissions at Southwest Research Institute has for a number of years conducted engineering studies for both industry and the EPA. These studies have included many types of engines categorized as both mobile and stationary source emitters. These studies have been directed at determining the effects of engine modifications on exhaust emissions and the impact on the environment. One such program for the EPA involved two engines, a two-stroke cycle and four-stroke cycle diesel, which were run on dynamometers on specified duty cycles for extended time periods.

We in the Department of Environmental Sciences were asked to provide support for this program in the preparation of samples for biological, in-vitro, toxicity testing. Also, the application of various analytical characterization methods was to guide the preparative efforts and provide information for preliminary identifications of compounds or compound groups which might be responsible for any toxic effects noted.

The objectives of the characterization phase of this study were to:

1. sample a large amount (200 g) of diesel engine exhaust particulates,
2. separate organic compounds from the particulate matter,
3. submit the organics for in-vitro toxicity testing, in particular the Ames mutagenicity test, and
4. provide analytical information for the identification of compounds.

The decision to collect a large amount of particulates was based on the need to have enough material for the tests which were to be conducted which included the Ames test and several other mammalian cell tests. These would require large amounts of material for each culture which is run at varous concentration levels. We also knew that the organic compounds extracted from particulates would be many in number and complex so we anticipated fractionating into functional groupings that would simplify the analytical work and make toxicity testing more meaningful. Material would also be needed for the analytical characterization work.

The particulates were deposited onto a fluted 30 cm x 300 cm glass fiber filter contained in a "backpack" holder through which the whole raw exhaust passed directly. Organics were extracted into dichloromethane (DCM) followed by extraction into acetontrile (ACN). The DCM extracts were then fractionated into acids, bases, and neutrals by liquid-liquid partitioning. The neutrals were subfractionated by column liquid chromatography on silica gel adsorbent. All of the fractions and the two extracts were submitted for biological testing as enough material became available during the course of the extractions and fractionations. Analytical characterization of the various fractions consisted of evaporating the solvent and weighing the residue; C, H, N determinations; infrared spectroscopy; and selective detection gas chromatography.

The two-stroke cycle engine exhaust deposited 3-4 g particulates on each filter and the other engine 2-3 g particulates. In preparation for extraction two filters were laid lengthwise one over the other and offset 8-10 cm. The uncovered flap of the lower filter was carefully folded over the upper one and the two were rolled into a cylinder 12-17 cm in diameter by 30 cm long. The filter was placed in a glass Soxhlet thimble which had been constructed by a glassblower since they are not commercially available. It took practice to roll the filters properly - if the cylinder was too large it would not fit in the thimble or if it was too tight the extraction solvent would not make good contact with the particulate sample.

The Giant Soxhlet extractors used are available commercially from Ace Glass Co. and we had one available. To get better than three months delivery we had three others made by Houston Glass Fabricating Co. None of these extractors would siphon with either DCM or ACN. We effected proper siphoning action by placing fluorocarbon tubing inside and pushing it to the top of the siphon tube and then placing a smaller diameter tube inside it. As with rolling the filters this operation was an art and it required a great deal of experimentation to find the proper size tubing that would provide proper siphoning.

The 12 l flask was charged with 5-6 l solvent and run for 24 hours at a cycle time of 70-90 minutes which resulted in 14-17 cycles/run over about 22 hours with the remaining time being given to take-down and start-up. When all of the filters in an engine series had been extracted with DCM the drained filters were then extracted with ACN. Extract batches from four filters were concentrated and the solvent recovered for reuse by distillation. The concentrates were then evaporated to dryness by inert gas impingement, and the residue was weighed and taken up in DCM for storage at -20°C.

First attempts to separate the acids, bases and neutrals were hampered by the formation of emulsions between DCM and water, and we eventually replaced the DCM with diethyl ether. The DCM containing 10-15 g of sample was evaporated; the residue taken up in 250 ml ether was extracted with 0.1 NKOH which was made acidic with H_3PO_4 and the acids were then extracted into ether. The ether solution was dried, weighed and stored in DCM solution as the ACD fraction. The remaining ether solution was extracted with 1 N H_3PO_4 and the basic compounds (BAS) were extracted into ether from the solution made basic with KOH. The compounds remaining in the original ether solution were named the neutral fraction (NUT). During the partitioning processes some tars formed and were deposited on the walls of the separatory funnels. Originally this material was recovered and stored as fraction INT but we found later that most of it could be dissolved by successive washings with the partitioning solvents. In some few cases the insoluble material from the four-stroke cycle engine would not completely redissolve.

The neutral group contained most of the extracted organics and was still quite complex so it was further fractionated on a column of silica gel primarily to separate the aliphatic hydrocarbons present in large quantities but which are not expected to exhibit any appreciable toxicity. The NUT solution containing approximately 4 g of the two-stroke or 2 g of the four-stroke sample was evaporated to near dryness and mixed with 1 g silica gel.* A hexane slurry was made and transferred to a 25 mm diameter glass column packed with 66 g silica gel which had been activated by heating 18 hours at 600 C. With this treatment the silica gel could be reused indefinitely without any adverse effects on the separations.

The aliphatic hydrocarbons (PRF) were eluted as the first fraction with 225 ml hexane. The end point can be determined by monitoring the Schlieren patterns in the column under visible light. Also, material fluorescent under long wave length UV reaches the bottom of the column at this point, and the eluant is changed to 1% diethyl ether in hexane. The aromatic fraction (ARM) is then collected in about 800 ml eluant, the cut off point being signaled by a narrow, yellow, nonfluorescent band reaching the bottom of the column. The yellow band containing the "transitional" compounds (TRN) is then collected in 400-450 ml eluant which is then changed to acetone/methanol (1:1). About 200 ml removes the oxygenated fraction (OXY), a dark orange-brown material which has to be chased with 200 ml methanol to remove the remaining tint from the column.

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* Bio-Sil A, 100-200 mesh, available from Bio Rad Laboratories, Richmond, California

The fuel used in these engines was also chromatographed on silica gel. The fuel did not contain any acidic or basic compounds measurable by gravimetric determination so 2 g was chromatographed neat. The results of fractionation of the fuel and organic-soluble particulates are shown as Table I. Apparently, most of the compounds comprising the fuel are aliphatic and small ring aromatic. The results are calculated as a percentage of the weight of total sample taken, exhaust particulates or liquid fuel. The differences in amounts and types of organics produced by the two engines are quite obvious in this table and become even more so in subsequent analytical determinations.

Elemental determinations were made on each fraction and the results are shown as trends of C/H ratios in Figure 1. The graph is a good preliminary indicator of the character of the exhaust organics. Generally, as the C/H ratio increases substitution of heteroatoms and aromaticity increases. Also, lower ratios for the two-stroke cycle engine (I) fractions indicate the greater aliphatic character of the compounds in these groups.

The infrared spectrum was determined from 1 mg of each fraction on a KBr disc. A summary of the results is shown as Table II with interpretive comments alongside the major absorption bands. These results were expected for the most part but there is a large degree of aliphatic character indicated as present but not wholly anticipated in the transitional and oxygenated fractions. The greater cyclic and aromatic character of the four-stroke organics indicated by the carbon-hydrogen ratios is emphasized by the infrared results.

Each of the fractions from the engine exhausts was gas-chromatographed on 3% OV-17 liquid phase with several detectors of varying selectivity. The flame ionization gas chromatograms of the NUT from the two engines are shown in Figure 2. It can be seen that these two groups of compounds are very different both qualitatively and quantitatively. The chromatogram from each of the engine fractions was essentially identical with the corresponding whole neutral fraction shown here. Most of the compounds from Engine I elute early in the lower molecular weight region between the indexes at C_{14}-C_{24}; the Engine II compounds are concentrated in the higher molecular weight region between C_{24}-C_{40}.

Figure 3 shows chromatograms resulting from using a sulfur-

TABLE I. RESULTS OF FRACTIONATION OF DIESEL FUEL AND ORGANIC - SOLUBLE PARTICULATES

	% TOTAL WEIGHT		
FRACTION	2-STROKE	4-STROKE	FUEL
DCM	63.0	25.0	--
ACN	9.0	9.9	--
ACD	0.8	2.1	--
BAS	0.06	0.2	--
INT	----	2.1	--
NUT	61.3	20.2	--
Loss P	0.2	0.1	--
PRF	37.1 (60.5)	8.6 (42.6)	74.0
ARM	8.8 (14.3)	0.9 (4.4)	21.5
TRN	3.5 (5.7)	1.2 (5.9)	0.7
OXY	9.2 (15.0)	7.0 (35.6)	0.3
Loss F	2.6 (4.2)	2.5 (12.4)	3.5

TABLE II. INFRARED SPECTROSCOPY OF SOLUBLE-ORGANIC FRACTIONS

FRACTION	ABSORBANCE, cm^{-1}	REMARKS
PRF	2915,2850,1460,1375	aliphatic mixtures of petroleum orgin
ACD	3700-2300 broad 1700 broad 1600 1280,1225	O-H stretching C=O aromatic C=O stretch C-O stretch
BAS	3400 very broad 1700 strong, broad	hydrogen bonded N-H stretch possibly amide C=O
ARM	1600 strong 810,750 880,830	C=C stretch C-H bending v. strong in 4-stroke additional C-H in 4-stroke
TRN	2915,2850,1460,1375 1720,1690	aliphatic, very similar to PRF carbonyl, largely aliphatic in 2-stroke; conjugated, unsaturated and aromatic keytones in 4-stroke
OXY	1710,1600sh 1790 1600-1790	very similar to TRN aromatic ketones acyclic esters in 2-stroke; aromatic ketones and esters, γ- and δ-lactones in 4-stroke

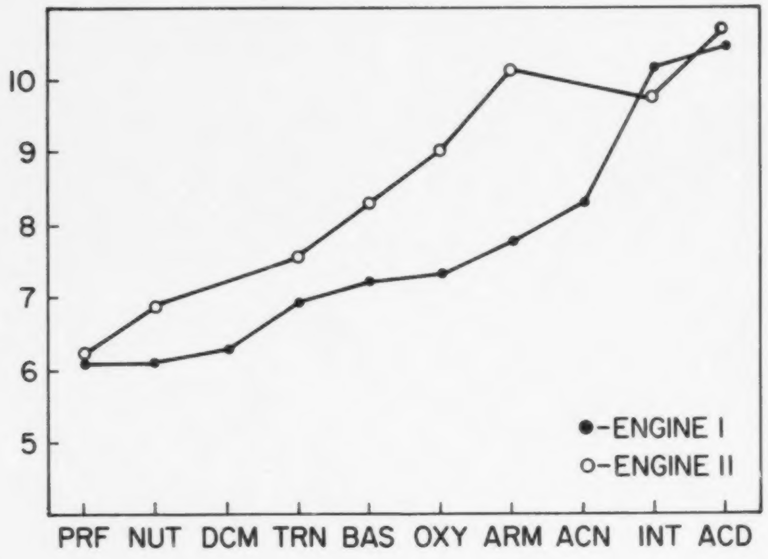

FIGURE I. CARBON/HYDROGEN RATIOS OF ORGANIC SOLUBLE DIESEL EXHAUST PARTICULATE FRACTIONS.

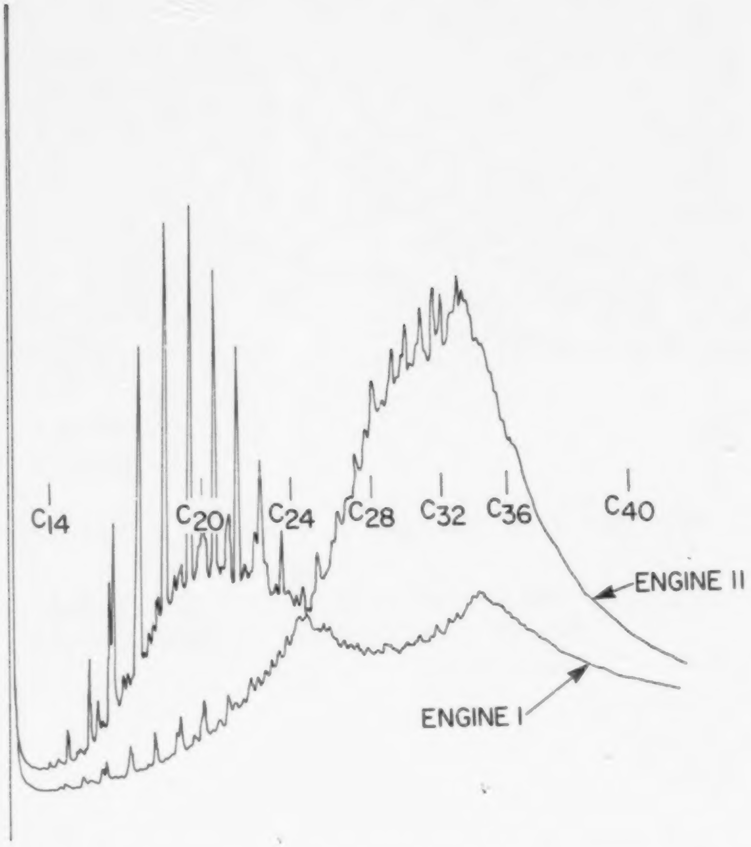

FIGURE 2. FLAME IONIZATION GAS CHROMATOGRAMS
OF NEUTRAL FRACTIONS.

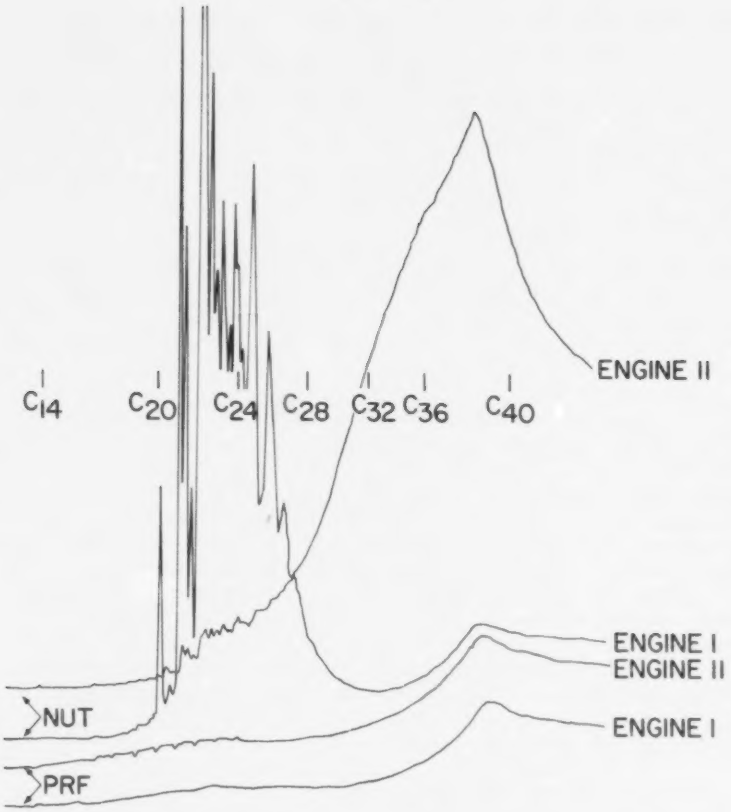

FIGURE 3. SULFUR-SELECTIVE FLAME PHOTOMETRIC GAS CHROMATOGRAMS OF NEUTRAL AND ALIPHATIC FRACTIONS.

selective Flame Photometric Detector. The chromatograms of the neutral fractions are not unlike those exhibited with the flame ionization detector except that they are shifted approximately four carbon units to longer retention times. The aliphatic fractions, as expected, indicate no detectable sulfur content. The transitional and oxygenated fractions from Engine II had some individual peaks superimposed on the envelope shown here for NUT in the region between C_{30} to C_{38}.

At this stage of the program we had enough of each of the fractions from the DCM extracts to begin submitting them for biological in-vitro testing. The most important results were to be those of the Ames mutagenicity tests conducted by Dr. Vincent Simmon at SRI International. The results of these tests were to guide the direction that further analytical characterization was to take. Other tests run were in-vitro mammalian cell assays by Dr. Martin Meltz, Southwest Foundation for Research and Education, unspecified tests, Dr. Leonard Schechtman, microbiological associates and *Drosophila* fruit fly screening, Dr. Ruby Vallencia, University of Wisconsin. The results of the biological tests were reported to Drs. Ronald Bradow and Mike Waters, EPA, Research Triangle Park, North Carolina. The C, H, N determinations were made by Galbraith Laboratories in Tennessee, HPLC and inorganic ion chromatography was conducted by Dr. Sylvestre Tejada at EPA-IERL, North Carolina, and some preliminary capillary GC/MS and high resolution MS by Dr. Evan Horning, Institute for Lipid Research, Houston, Texas.

The results of the Ames mutagenicity tests in decreasing order of activity are, TRN II, OXY II, TRN I, OXY I, ACD II, ACD I, INT II, BAS II, ARM II, ARM I. The remaining fractions, PRF I and II, BAS I, and INT I showed no activity. Activity is apparently concentrated in fractions containing aromatic/oxygenated functionalities and seems to be greater in the fractions from the four-stroke cycle engine. The results from the basic and insoluble tars fractions are ambiguous because of the very limited amounts which were available. These test results directed our analytical efforts to concentrate on the transitional and oxygenated fractions from the two engines. Gas chromatographic/mass spectrometric determinations were then begun on these fractions and some of the results are shown as Figure 4.

It was apparent that packed column gas chromatography would not provide very useful mass spectra of the individual compounds without a great deal of time and effort expended on computer manipulations of the data. We then attempted further fractionation of these groups of compounds by a "quick and dirty" procedure which follows:

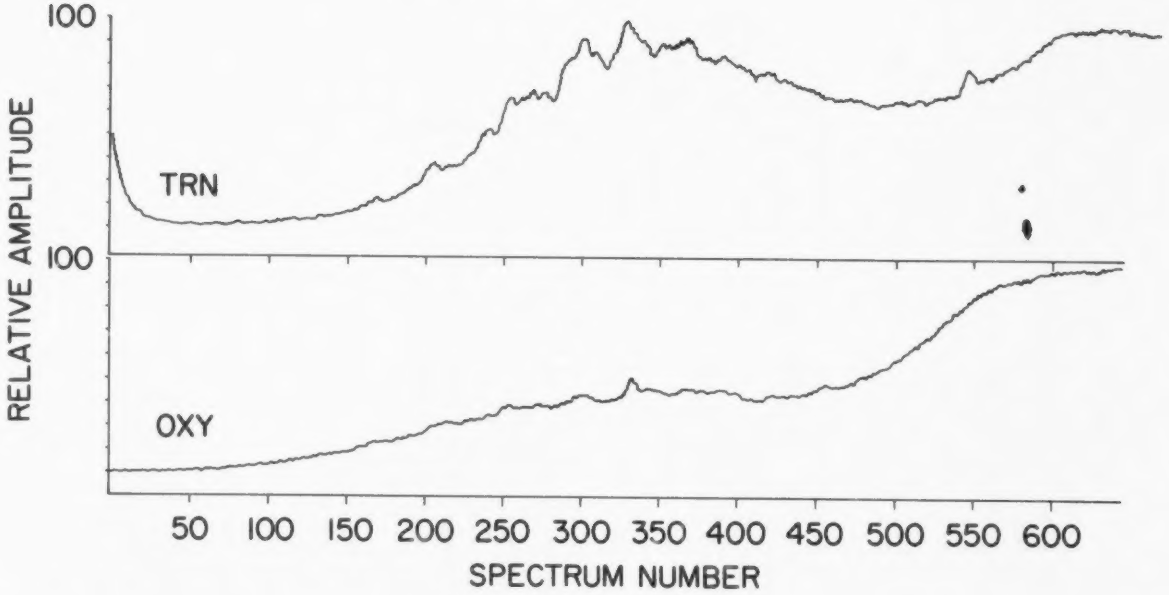

FIGURE 4. TOTAL ION CHROMATOGRAMS OF TRANSITIONAL AND OXYGENATED FRACTIONS FROM ENGINE II.

1. soak Sephadex LH-20* in iso-octane, methanol, chloroform,
2. pack swollen LH-20 gel in a 100 mm (l) x 4 mm (i.d.) glass column to a height of 40 mm,
3. place 1-1.5 mg sample in 250 μl solvent on the LH-20 column,
4. elute with the solvent mixture, catch five 1 ml fractions, and
5. elute fraction 6 with 20 ml methanol.

The glass column in Step 2 is a Pasteur pipet with the tip cut off at a convenient point. Nitrogen at a very low pressure (<5 psig) was applied to the head of the column to hasten elution of the solvent.

GC/MS of the subfractions from the gel chromatography provided chromatograms that were much more useful than those from the whole fractions. Some of these chromatograms are shown as Figure . The peaks are more discretely separated, but the "envelope" which results from the incomplete separation of many similar compounds is still present. The same characteristics of earlier chromatograms are again evident, i.e., the two-stroke exhausts are made up of earlier eluting compounds of low molecular weight.

Useful mass spectra could be extracted from these total ion chromatograms with more ease than from those of the whole fractions. Interpretable spectra were generated by careful choice of the peaks for study and by subtraction of background taken from both the leading and trailing sides of the peak. These spectral enhancement manipulations resulted in the tentative identification of a number of compounds, as follows:

alkyl naphthalenes (ARM I, II)
anthracene/phenathrene (ARM I, II)
alkyl anthracene/phenanthrenes (ARM I, II)
fluoranthene (ARM I)
pyrene (ARM I)
benzofluorene and/or methylpyrene (ARM I)
o-phenyl anisole (TRN II)
4-methylphenylbenzo(c)cinnoline (TRN II)
nitropyrene (TRN II)
phenylethylketone (OXY I)
chromone (OXY I)
coumarin (OXY I, II)
formylnaphthalene (OXY I, II)
alkyl naphthols (OXY I, II)

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*A cross-linked dextran gel available from Pharmacia Fine Chemicals, Piscataway, New Jersey

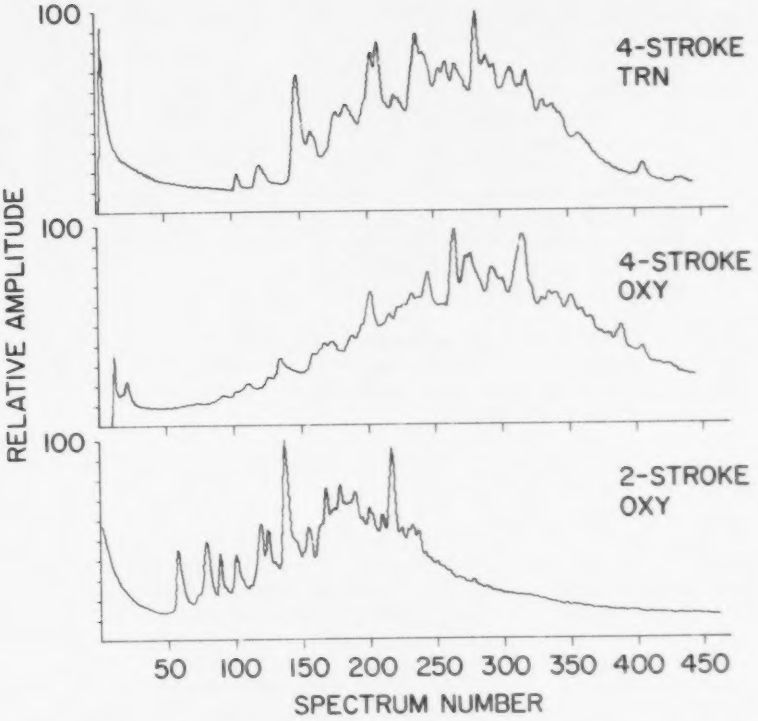

FIGURE 5. TOTAL ION CHROMATOGRAMS OF DIESEL ORGANIC SUBFRACTION 4

perinaphthindenone (OXY I, II)
anthroquinone (OXY I, II)
benzanthrone (OXY I, II)
m-hydroxybenzaldehyde (OXY I, II)
methoxybenzaldehyde (OXY I)
naphthol (OXY I, II)
p-phenylphenol (OXY I, II)
dibenzo-p-dioxin (OXY I, II)
naphthoic anhydride (OXY I, II)
o-phenylphenol (OXY I)
methylbenzanthrone (OXY II)
anthrone (OXY II)

The fractions each compound was indicated to be present in are indicated in parentheses. For example, (OXY I, II) indicates the spectrum was found in the oxygenated fraction from both engines.

The analytical characterizations were secondary to the main purpose of this program; hence, were of preliminary and supportive nature. The primary objective of providing a large amount of diesel engine exhaust particulate organics in useful compound groups for biotoxicity testing was accomplished. The fractionation methods described may prove useful for continuing studies of the biological effects of diesel exhaust constituents and determination of the compounds responsible for toxic effects. These studies could include more definitive determinations of toxicity, determination of the fate of exhaust compounds as they are emitted to the atmosphere, mammalian exposure and inhalation experiments, and the development of methods of eliminating unwanted compounds from diesel exhaust.

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A RAPID CHEMICAL CHARACTERIZATION OF DIESEL PARTICULATES BY THERMOGRAVIMETRIC ANALYSIS

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ABSTRACT

Thermogravimetric analyses, carried out on particulate produced by an oil flame and by a diesel engine, allow the determining, in a short time, of the volatile content associated to the particulate and the distinguishing of the unreacted fuel and/or lubricant oil from the combustion products.

Thermogravimetric values, compared to those obtained by solvent extraction, display that the extraction procedure usually employed was not complete and in some cases the extracted organics account for about 50% of the volatile content of the particulate. These results are confirmed by thermogravimetric analysis of particulate after solvent extraction.

The thermogravimetric technique is compared and integrated by direct gas chromatographic and mass spectrometric analysis of particulate before and after solvent extraction.

INTRODUCTION

Sampling and collection, chemical and physical characterization, health effect evaluation are the three main steps involved in diesel particulate emissions. The complexity of the different problems related to these three steps requires a very long time to obtain significant experimental evidences. Thus, the possibility to achieve a first rapid information about particulate exhausted by diesel engines would be welcome. This is particularly true for the manufacturers who need a quite short assessment in order to test the influence of technological engine modifications on quality and quantity of the organics associated to particulates.

The aim of the present paper is to face with the thermogravimetric characterization of the organics in order to obtain a short answer to the main engine parameter changes. At the same time the need for particulate collection and the possibility for the health effect studies following this rapid chemical characterization are examined.

At first this new method has been verified on the steady-state pilot burner due to the better possibility of obtaining large amounts of particulate. Then it has been extended to diesel particulates and preliminary evidences will be shown.

For the better understanding of the chemical composition of the organics the new system has been compared and integrated by direct gas chromatographic and mass spectrometric analysis of particulates.

EXPERIMENTAL

Samples

The measurements have been carried out on two series of particulates. The first was produced by an oil flame (described in detail in a previous paper) (1), that presented properties similar to those that may be found in residential and industrial burners. The picture of Fig. 1

shows the oil flame. Two particular zones can be distinguished along the axis: the first one a spray zone, the second one a combustion zone. The sampling was made by means of a sintered bronze filter placed inside a water-cooled stainless steel probe, that can be moved along three axes of the flame to investigate on the droplets vaporization and combustion and particulate formation and evolution at different stages of the combustion.

The second consists of particulate samples exhausted from a N/A 2.4 l displacement 4-cyl IDI engine, with and without oxidation catalyst, tested under 1975 Federal Test Procedure-Urban Driving Dynamometer Schedule, and collected according to EPA proposed procedure (2).

Procedure

Thermogravimetric measurements of particulates were carried out using a Perkin-Elmer TGS-2 Analyzer (Fig. 2). A detail of furnace and balance is shown in Fig. 3. The samples, 5 ÷ 10 mg, were put in a pan platinum inside a furnace heated up to 600° C. The heating rate of the furnace, 10° C/min, was controlled by a Model UU-1 temperature program control. The furance atmosphere was controlled by a flow of purified helium (SIO UPP type, 99.998%, 10 ml/min) at atmospheric pressure. The first derivative, weight loss versus temperature (dW/dT), was diagrammed by a model FDC-1 derivative computer. The thermogravimetric method was firstly calibrated with known syntehtic mixtures of diesel fuel and/or lubricant oil on carbonaceous support.

Figs. 4 and 5 show some examples of thermogravimetric curves of a particulate, the first containing 10.7% diesel fuel only, and the second 33% diesel fuel and 13% lubricant oil.

Quantitative values of unreacted fuel and/or lubricant oil in particulates were obtained measuring the area of the peaks of the first derivative with a maximum at about 150° C for unreacted fuel, and 300 °C for lubricant oil.

The gas chromatographic analysis of particulates was carried out by a direct sampling of solid samples, 1 ÷ 5 mg, in the injection block of a gas chromatograph (C. Erba mod. GI) kept at 450° C, by a solid injector apparatus (3). The volatile products were separated by a stainless steel column, 2 m x 2 mm, packed with 5% Dexsil-300-GC on Chromosorb W, 80 ÷ 100 mesh. The column temperature was isotherm for 5 min and then programmed linearly to 370° C at 6° C/min; helium was used as carrier gas, at 10 ml/min.

Figure 1. Oil flame.

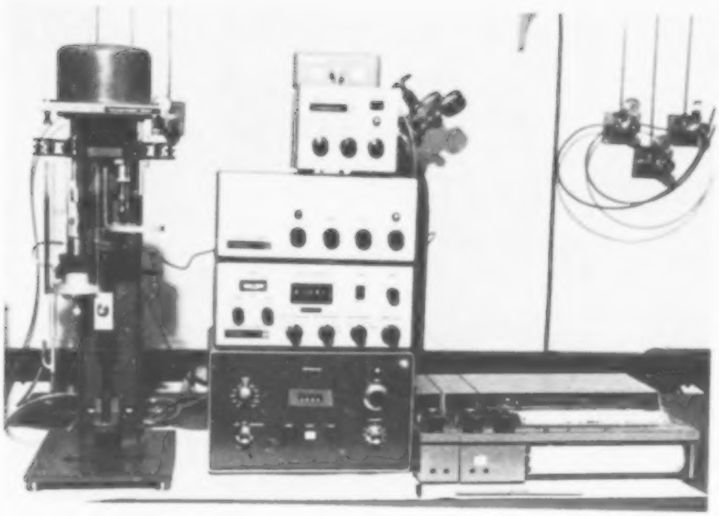

Figure 2. Thermogravimetric apparatus.

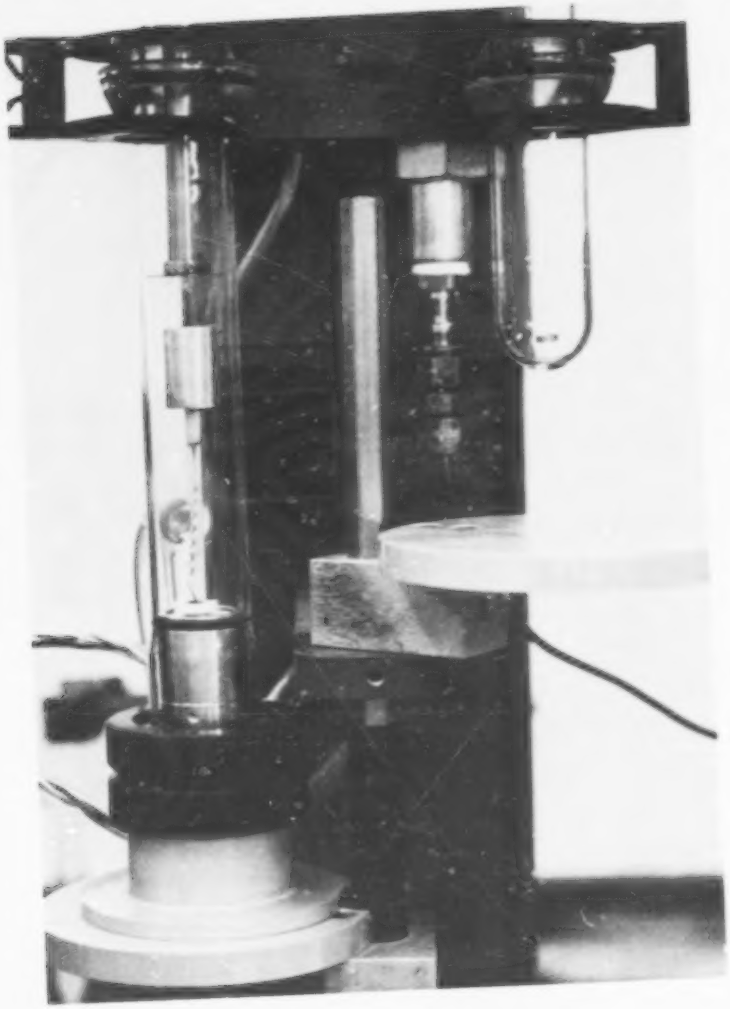

Figure 3. A detail of furnace and balance of the termo-gravimetric apparatus.

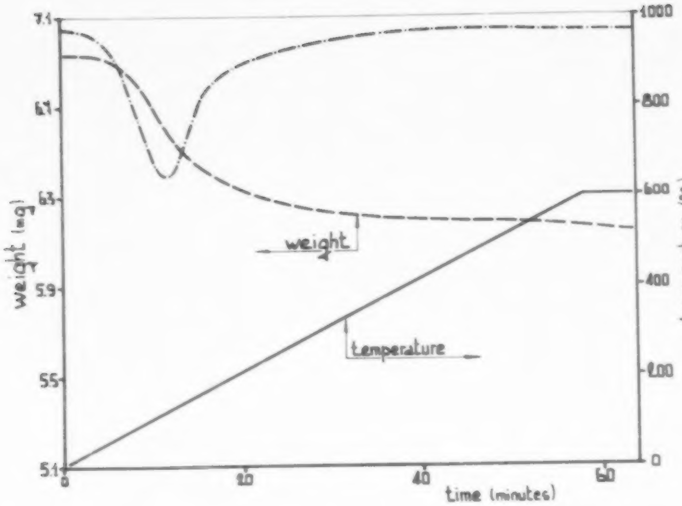

Figure 4. Thermogravimetric and first derivative curves of a syntetic mixture containing 10.7% diesel fuel on carbonaceous support.

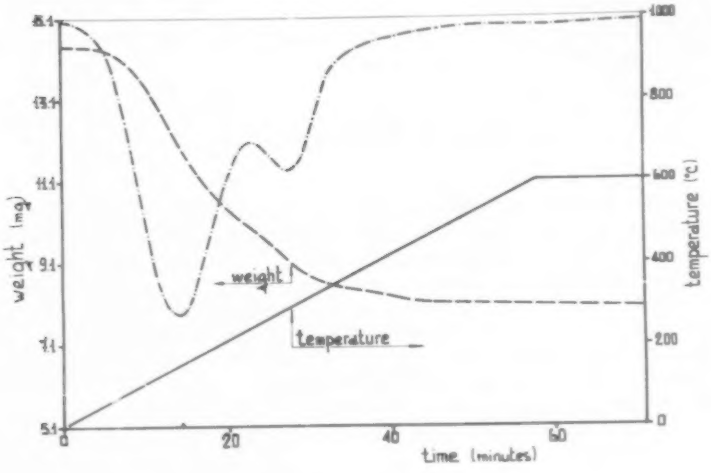

Figure 5. Thermogravimetric and first derivative curves of a syntetic mixture containing 33% diesel fuel and 13% lubricant oil on carbonaceous support.

The particulates were also analyzed by direct sampling in a mass spectrometer with a data system in line (4-5). The samples, about 0.1 mg, were placed in a quartz capillary at the tip of a solid probe. The probe was then inserted into the mass spectrometer and heated up to 500° C, with an automatically controlled temperature programmer, at about 40° C/min. An EI source with electron energy at 70 eV was used.

The extraction and separation of the organic components present in both types of particulates were carried out according to the EPA procedure (6): the particulate, collected in repeated runs in different filters, was placed in batches in soxhlet thimbles (previously, extracted for 6 hr with dichloromethane) and extracted for 8 hr with dichloromethane (DCM).

The particulate after extraction was dried and used for further analysis by means of mass spectrometry and thermogravimetry. The extract material was evaporated to dryness and then weighted. This value was compared with that obtained by thermogravimetric analysis.

Then the dried extract was dissolved in diethyl ether and subdivided on a silica gel column into paraffinic (PRF), aromatic (ARM), transitional (TRN) and oxygenated (OXY) fractions. Samples of all the above fractions were prepared for in-vitro mutagenicity analysis (7). Part of TRN fraction was dried and analyzed by direct sampling in a mass spectrometer using a solid probe.

RESULTS AND DISCUSSION

The particulate samples include adsorbed uncombusted fuel (and/or oil lubricant in those emitted from diesel engine) and a variety of combustion products including some high molecular weight organics. The thermogravimetric analysis of these samples allows the determining in a short time of the volatile content associated to the particulate by weight loss versus temperature (T). Furthermore the first derivative of the TG curves (dW/dT) allows the distinguishing of the unreacted fuel, the lubricant oil and the combustion products capable of being vaporized up to 600° C in inert gas flowing.

Fig. 6 shows, for example, the thermogravimetric curves related to a soot sample collected immediately to the downstream spray zone of the oil flame. The total weight loss up to 600° C was 12.3% of which a large amount was in the temperature range betweeen 100° and 240° C. The first

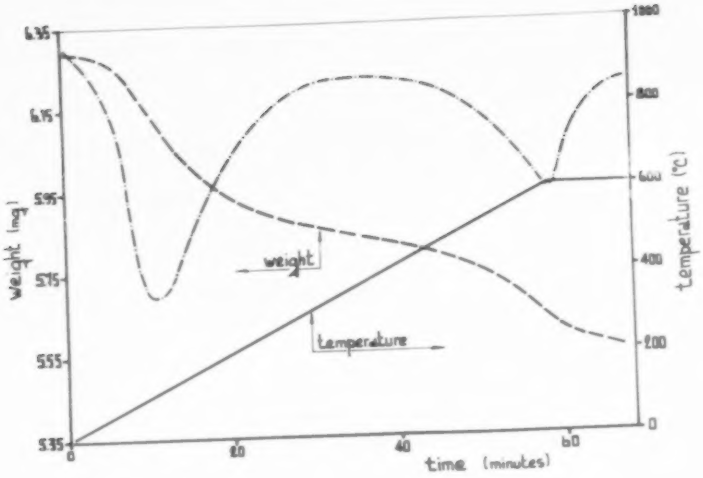

Figure 6. Thermogravimetric curves related to a soot sample collected immediately to the downstream spray zone of the oil flame. Total weight loss=12.3%.

derivative illustrates this result better: there is a very big peak with a maximum at 140° C and another evident peak has a maximum at about 600° C. The big peak was very similar to that obtained in calibration curves of diesel fuel on carbonaceous support and was attributed to the unreacted fuel; the percentage calculated of diesel fuel was 7.1%. Other analyses of particulate collected inside the spray zone of the oil flame allowed the tabulation of the content of unreacted fuel and of combustion products, and the possibility of having more detailed information on the structure and properties of the spray combustion of liquid hydrocarbons. The TG results, moreover, were compared with those obtained on optical measurements based on laser light scattering used to distinguish fuel droplets and particulates in the spary zone (8). From the comparison of the first results it seems that these two techniques together can give a more detailed information on particulate formation in practical combustion devices.

Fig. 7 illustrates the thermogravimetric curve and the first derivative of soot collected in the combustion zone of an oil flame. The total volatile content was 3.3%; the peak related to unburned fuel had completely disappeared, while that of combustion products was particularly evident.

Fig. 8 illustrates the thermogravimetric curve and the first derivative of a particulate sample exhausted from a N/A 2.4 l displacement 4-cyl DI Diesel engine without oxidation catalyst. The organic content was 39%, of which the part evaporated up to 250° C accounts for about 50%. The first derivative shows the peak of the unburned fuel and that of combustion products, less evident in this analysis was the peak of lubricant oil, with a maximum at about 300° C. There is also to note that the first derivative shows, at low temperature, the presence of compounds produced from the combustion process, by the cracking of higher molecular weight materials.

Fig. 9 shows the thermogravimetric curve and the first derivative of particulate sample exhausted from the same N.A. Diesel engine of Fig. 8 with oxidation catalyst. The total volatile content in this soot was less than that of Fig. 7, 17.6%. The distribution of unreacted and combustion products is particularly evident from the first derivative: the peak with a maximum at 60° C gives evidence of the cracking products from higher molecular weight materials, the peak at 180° C of unreacted fuel, the peak at 300° C of lubricant oil, the last peaks of the combustion products.

For a more significative characterization of the TG peak of unreacted fuel associated to particulates, we have analyzed soot samples as collected by direct sampling in a gas chromatograph.

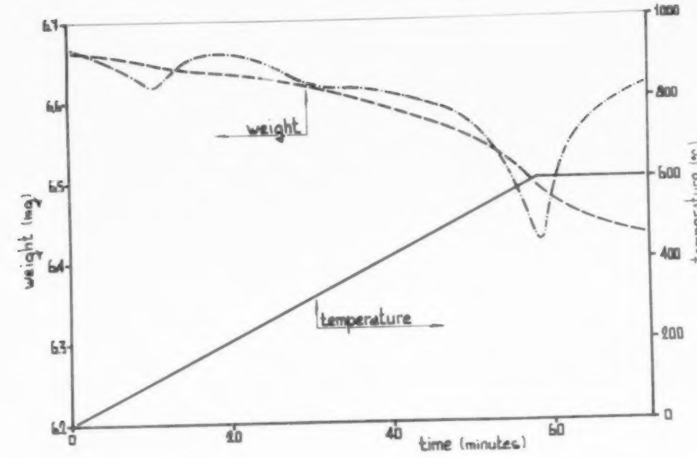

Figure 7. Thermogravimetric curves related to a soot sample collected in the combustion zone of the oil flame. Total weight loss=3.3%.

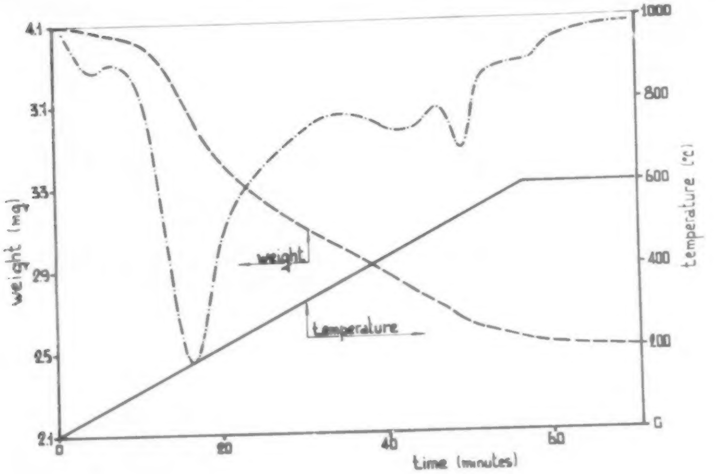

Figure 8. Thermogravimetric curves of a particulate exhausted from a diesel engine without oxidation catalyst. Total weight loss=39%.

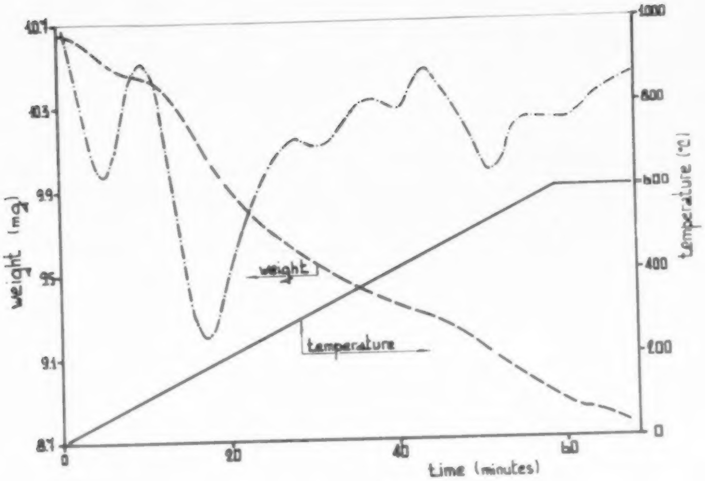

Figure 9. Thermogravimetric curves of a particulate exhausted from a diesel engine with oxidation catalyst. Total weight loss=17.6%.

Figs. 10 and 11 show the gas chromatographic results for particulate collected inside the spray zone of the oil flame and of the fuel, respectively. The distribution of peaks is about the same, this means that the attribution of the TG peak at about 170° C to the unburned fuel was correct.

Several significant differences were noted, instead, in the gas chromatogram for particulate collected downstream of the spray zone, Fig. 12. The first part of the chromatogram can be ascribed to unreacted fuel, the second part shows peaks of combustion products; nevertheless, also in this soot the content of unburned fuel can be correctly calculated.

Particulate samples produced in spray oil flame at different heights along the vertical axis (that correspond to different stages of the combustion) and by Naturally Aspirated (NA) and Turbocharged (TG) engines, in some cases with oxidation catalyst (ox. cat.) and/or exhaust gas recirculation (EGR), were extracted with dichloromethane (DCM). Table 1 shows extraction and the volatile content determined as percentage of weight loss of soot as collected by thermogravimetric curves (TG1). TG1 values exhibit a volatile content of particulates much higher than those obtain by DCM extraction. This means that the extraction procedure was not complete and in some cases the extracted organics account for about 50% of the volatile content of the particulate.

In the same Table 1 can also be seen the thermogravimetric weight loss values of the particulate analyzed after DCM extraction (TG2). These values confirm that the DCM extraction was not complete. In the last line of Table 1 are shown the calculated differences between TG1 and TG2 (ΔTG). A good correspondence was observed between these values and those of DCM organic extract.

An example of thermogravimetric analysis of a particulate before and after DCM extraction is reported in Fig. 13. This particulate was collected immediately downstream of the spray zone of oil flame. The comparison shows that the higher compound content remains unextracted in soot particles.

For the characterization of this higher compound content, we have extensively analyzed the particulate samples, before and after extraction, by direct probe-mass spectrometer interfaced with a data system. In this connection particular attention has been devoted to the content of higher polycyclic aromatic hydrocarbons (PCAH).

The following experiments were carried out on soot produced in oil flame spray.

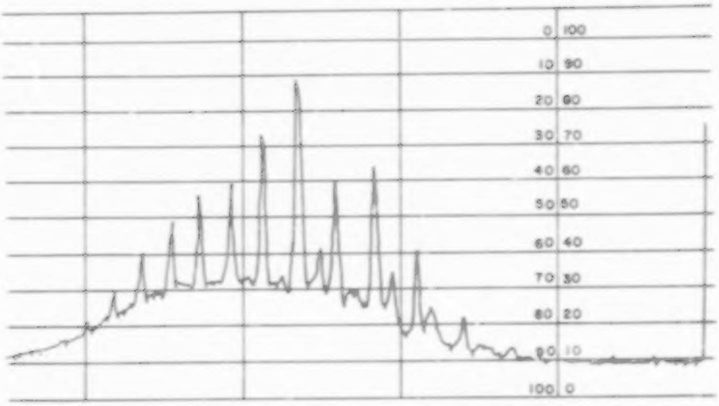

Figure 10. Gas chromatogram of a particulate collected inside the spray zone of the oil flame.

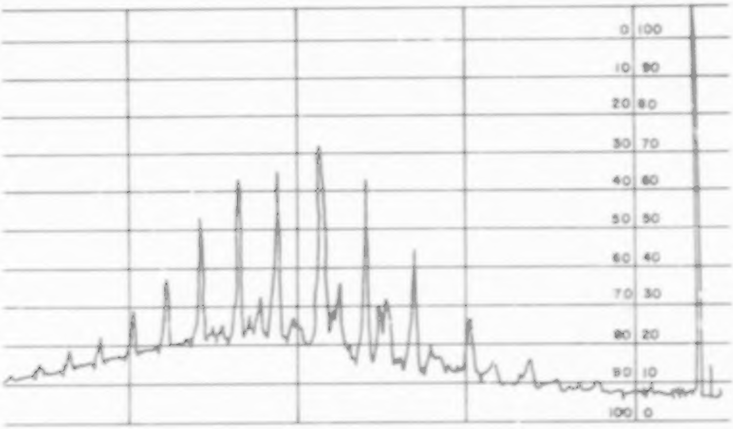

Figure 11. Gas chromatogram of the fuel.

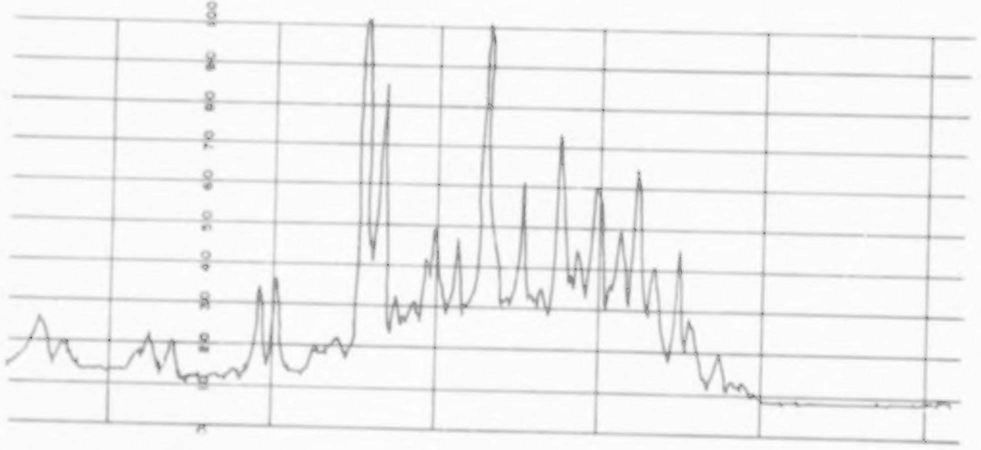

Figure 12. Gas chromatogram of a particulate collected downstream of the spray zone of the oil flame.

TABLE 1. DCM Organic Extracts and Thermogravimetry Analysis (% weight) related to Particulate collected from Different Zones of a Oil Flame and from Different Diesel Engine Configurations.
(TG1: Thermogravimetry before the extraction, TG2: Thermogravimetry after the Extraction, ΔTG: (TG1-TG2))

SAMPLES	Extract	TG1	TG2	ΔTG
Oil Flame	16.8	26.4	8.9	17.5
	9.0	16.0	6.3	9.7
	4.7	8.9	3.6	5.3
N.A.	29.5	51.8	22.1	29.7
N.A. +Ox.Cat.	16.5	34.0	16.1	17.9
T.C.	7.8	19.5	11.6	7.9
T.C.+ EGR	14.8	20.4	5.4	15.0
T.C. + EGR + Ox.Cat.	6.4	13.5	6.8	6.7

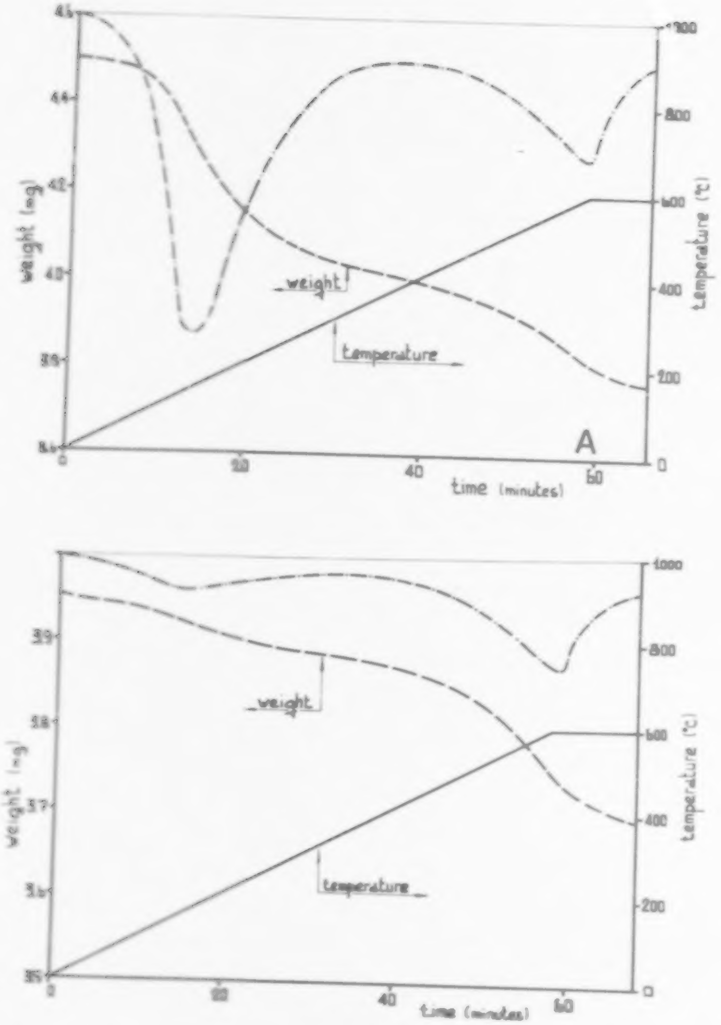

Figure 13. Thermogravimetric curves of particulate, before (A) and after DCM extraction (B). This particulate was collected immediately downstream of the spray zone of the oil flame.

Fig. 14 illustrates the mass spectrum related to the higher m/e ratios analyzing soot samples as collected. The spectrum shows clearly that compounds with high molecular masses are associated with soot: the most prominent ions are 350, 374, 398, 400, 424, 448 and 472, corresponding to some possible aromatic structure with from 8 to 11 rings.

Fig. 15 shows the profiles versus time (and temperature) of the total ion current (TI) and of single ions, attributed to PCAH, for soot collected in the combustion zone of oil flame, similar to that of Fig. 7. Of course, vaporization carried out under vacuum and a temperature programmed up to 500° C, at 40°/min, does not separate the compounds of the mixture one by one; nevertheless, the ability of the on line computer to store and follow the spectra taken during the analysis can help to give useful information and evalutions. Moreover, the profile of the total ion current, reproduces with good approximation the peak of the first derivative related to the combustion products of Fig. 7.

Fig. 16 shows the mass spectrum of the soot after DCM extraction. The spectrum confirms the information obtained by TG analysis about the presence of substances at higher molecular mass in soot after extraction and that the most prominent masses correspond to the higher PCAH.

Bearing in mind that the mass spectra of unsubstituted PCAH consists of a very intense molecular ion accompanied by a few weak fragment ions, it was possible to obtain a semi-quantitative determination of PCAH present in soot before and after DCM extraction.

Figs. 17 and 18 illustrate some examples of the profiles of molecular ions of different PCAH obtained by direct probe analysis of soot before and after extraction. The PCAH with lower molecular masses, such as 202, 226 and 252 (Fig. 17) present in soot before extraction are absent in soot after extraction. The result is different in the profiles of higher PCAH: the area of molecular ions 374 and 398 in soot after extraction (Fig. 18) represent a significative percentage as compared to that of soot before extraction and this percentage is higher for the ion 398.

An example of spectrum related to soot after DCM extraction is reported in Fig. 19. Also in this spectrum the presence of PCAH with higher molecular masses is evident.

The computer calculated areas under the peaks related to molecular ions of PCAH for soot before and after extraction are listed in Table 2, where the calculated area ratio percentages of ions are also reported.

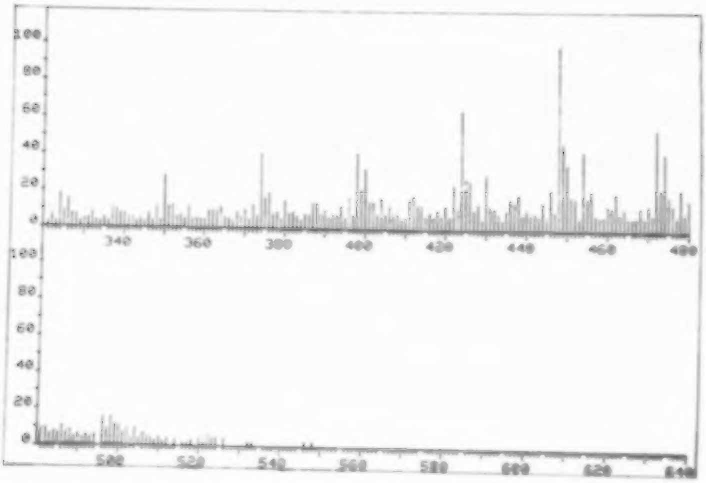

Figure 14. Mass spectrum related to the higher m/e ratios of soot collected in oil flame.

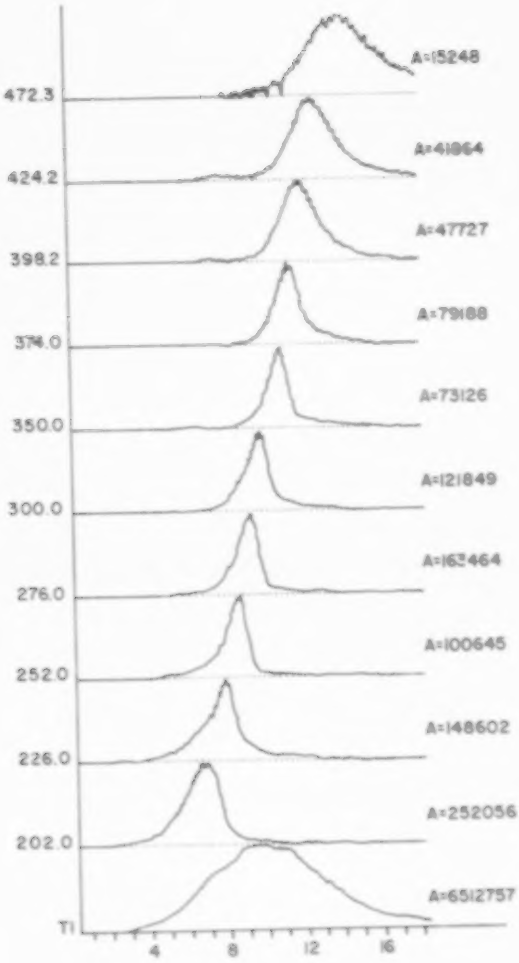

Figure 15. Profiles versus time (and temperature) of the total ion current (TI) and of single ions, attributed to PCAH for soot collected in oil flame.

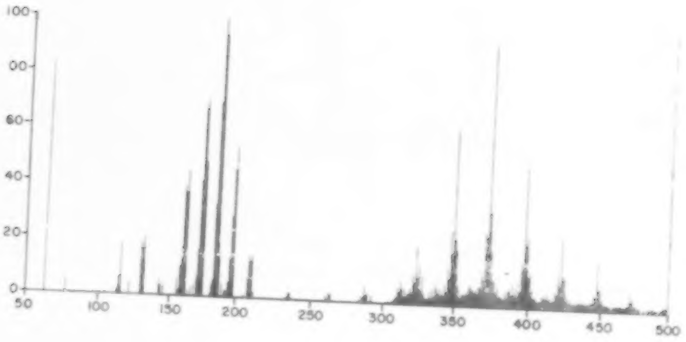

Figure 16. Mass spectrum of soot after DCM extraction.

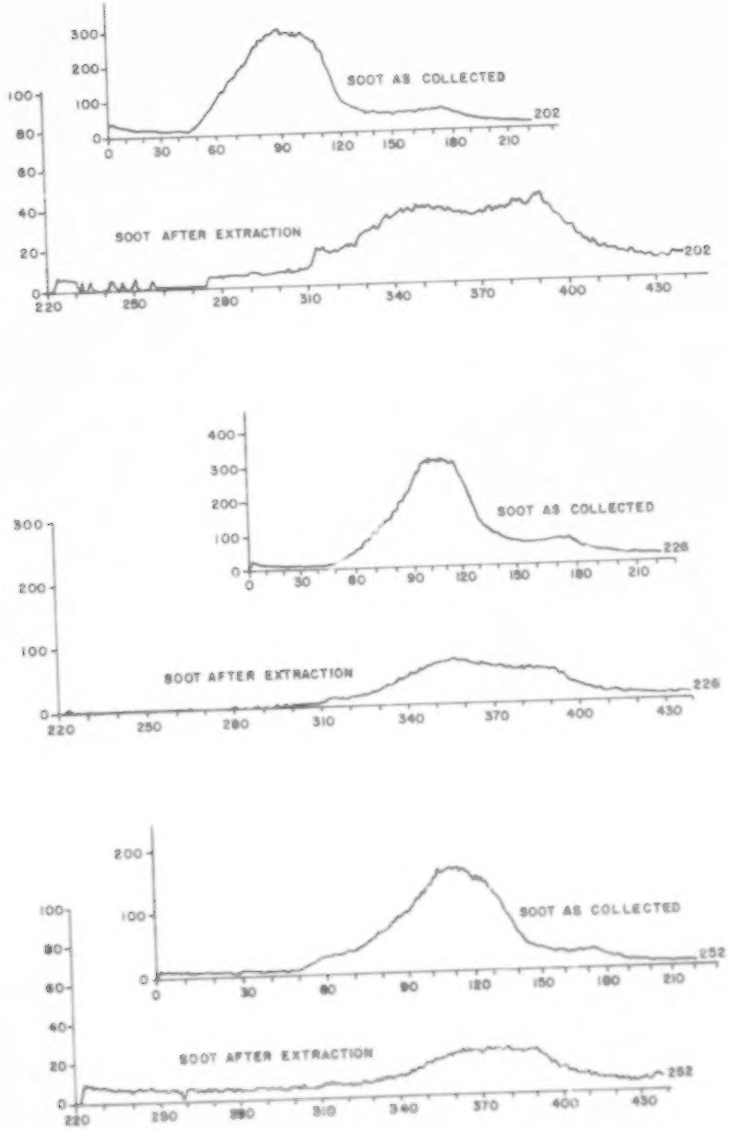

Figure 17. Profiles of molecular ions of PCAH at lower molecular masses obtained by direct probe mass spectrometry of soot before and after extraction.

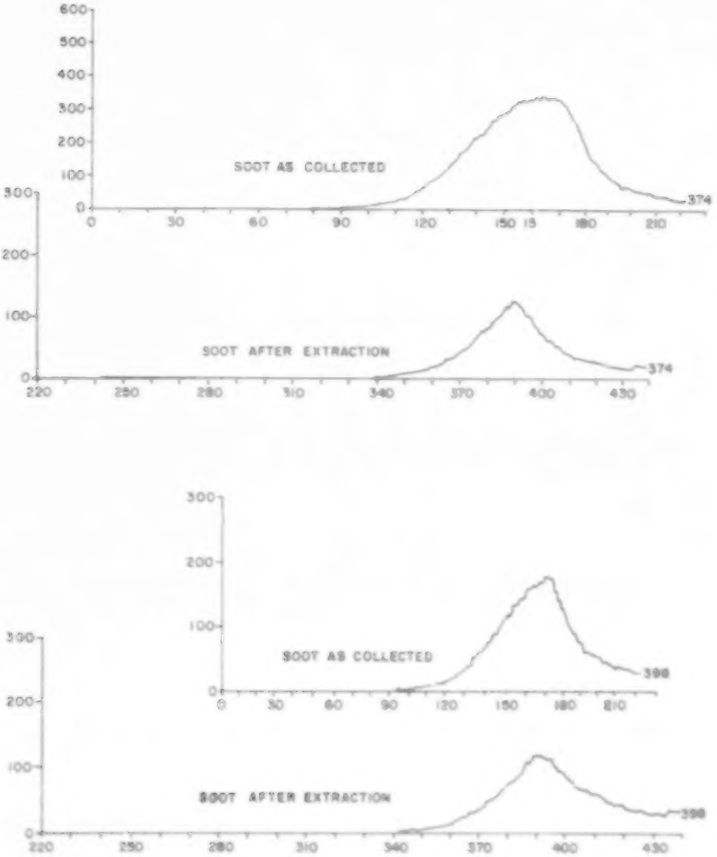

Figure 18. Profiles of molecular ions of PCAH at higher molecular masses obtained by direct probe mass spectrometry of soot before and after extraction.

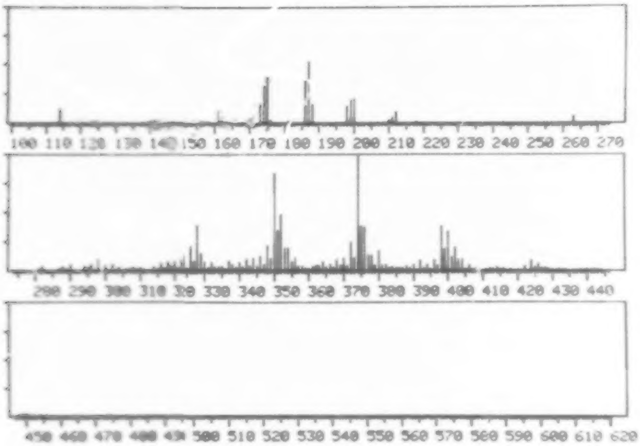

Figure 19. Mass spectrum related to soot after DCM extraction.

TABLE 2

Computer calculated area under the peaks of molecular ions of possible polyaromatic structure for soot as collected (A), for soot after extraction (B) and area ratio percentage of SOOT B / /SOOT A .

	SOOT A	SOOT B	Area ratio B/Ax100
m/e	area	area	
202	1613824	50605	3.0
226	1441792	4676	0.3
252	761856	8220	0.4
276	856064	35020	4.1
300	458752	32768	7.1
326	382976	88960	23.2
350	417792	137920	33.0
374	317440	125630	39.6
398	334144	224416	67.2
400	352880	226224	64.1
424	102080	87008	85.2
448	128160	107220	83.7
472	52250	42960	82.2

These values are almost negligible up to 300 m/e, and increase very quickly at higher m/e values, showing that the more incomplete DCM extration was the higher the molecular masses of PCAH were.

This significant lower yield of extraction is better depicted in Fig. 20. The efficiency in DCM extgraction from soot of unsubstituted PCAH from seven-ring polycyclic compounds (coronene) to ten-ring (ovalene) decreases rapidly down to very low values. In our opinion, it does not fall to zero at higher masses because these compounds are difficult to vaporize even when the soot was heated up to 500° C.

A much greater fraction of the high molecular weight PCAH extracted with DCM was present in the transitional fraction (TRN). This fraction was dried and then analyzed by direct probe mass spectrometry. Figs. 21-23 show the resulting mass spectra at different times of analysis of the fraction. Molecular ions of higher PCAH can be recognized in the spectra, such as 350, 374, 376, 398, 400. Figs. 24 and 25 show the profile of some molecular ions of PCAH present in TRN Fraction, and give an idea of their relative abundance.

There are some problems associated with the diesel particulate collection system usually employed. In fact filtration of diesel exhaust hinders to achieve a sufficient particulate amounts useful for a their complete chemical characterization by means of thermogravimetry, gas chromatography and mass spectrometry. The collection efficiency of filters recommended by EPA as printed out in references (9,10) and the plugging of filters resulting from the particulates going inside the filter pores does not allow to remove the particulate from the filter surface.

A collection system based on the thermal gradient effect appears the most promising in order to collect the very low size (< 0.2 μ) non conductive diesel particles with high efficient and in the same time to allow scraping the particulate on the thermal precipitator walls.

Preliminary mass spectrometric analyses of diesel particles have shown that there are a few differences between the combustion product distribution associated to these particles and that associated to particles collected in oil flame spray. The less controlled combustion in diesel engines generates a greater variety of combustion products, above all some partially oxidized organics.

However, we are also going to carry out analysis on diesel particulates, before and after extraction, in thermogravimetric apparatus working under high vacuum and interfaced with mass spectrometer. This combined system may allow to a

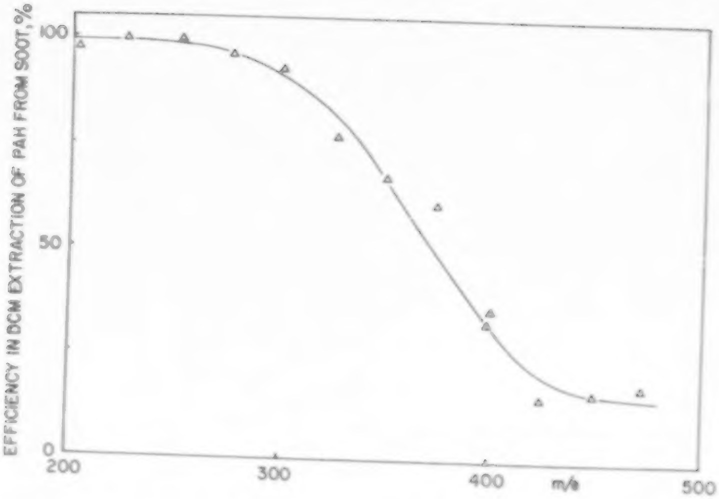

Figure 20. The efficiency in DCM extraction from soot of PCAH at different molecular masses.

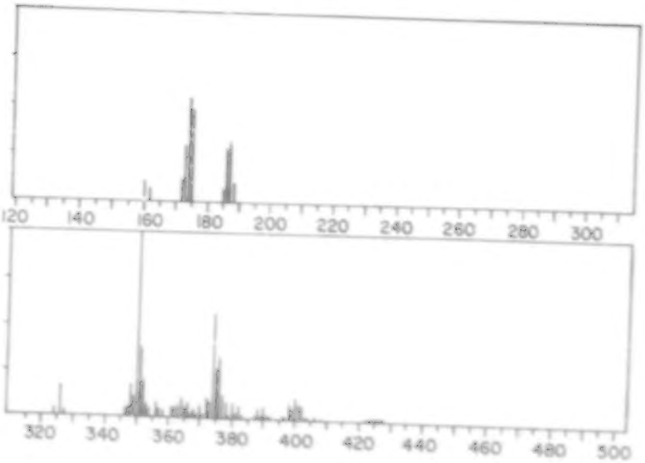

Figure 21. Mass spectrum 68 of the transitional fraction.

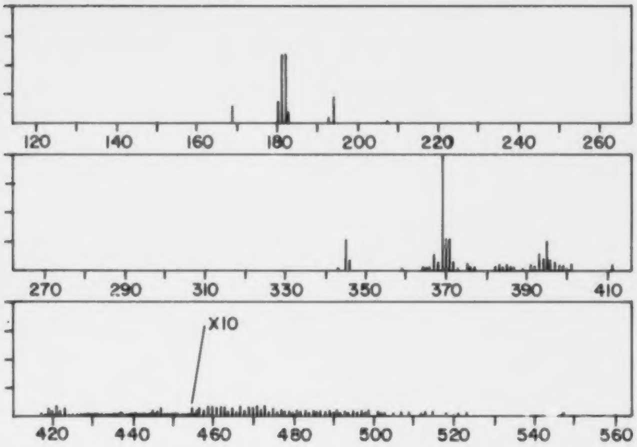

Figure 22. Mass spectrum 133 of the transitional fraction.

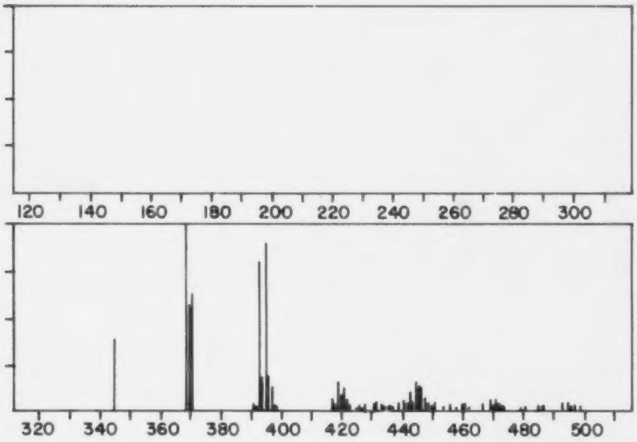

Figure 23. Mass spectrum 147 of the transitional fraction.

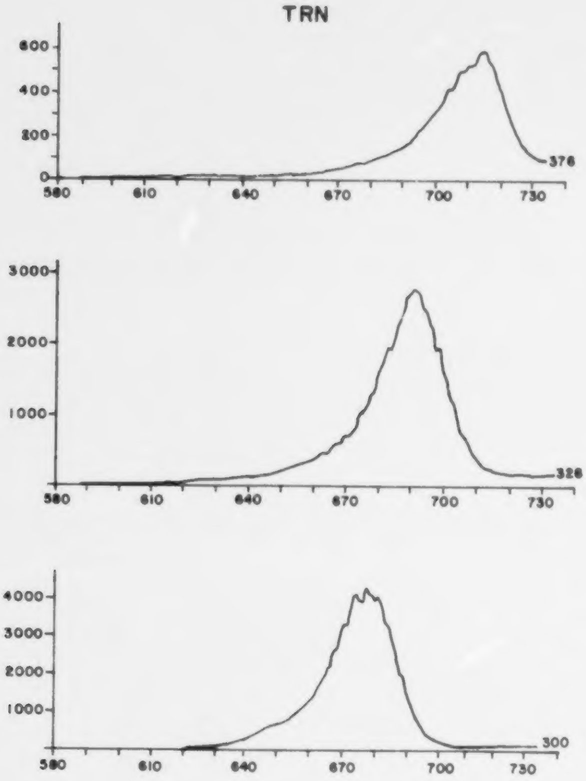

Figure 24. Profiles of molecular ions of some PCAH present in the transitional fraction.

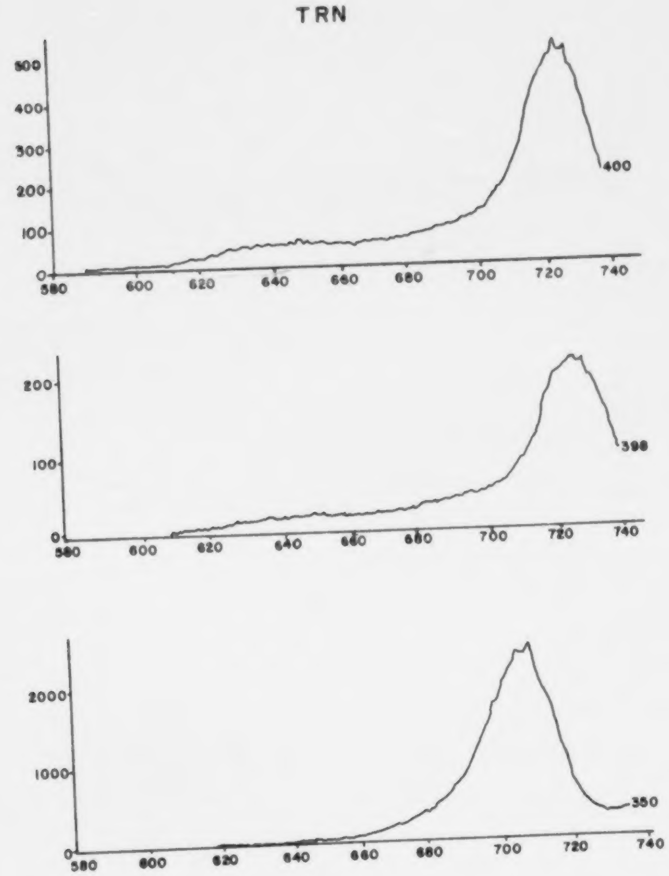

Figure 25. Profiles of molecular ions of some PCAH present in the transitional fraction.

direct and better characterization of the thermogravimetric curves and the first derivative peaks, and to carry out analysis in a very short time, without manipulations.

CONCLUSIONS

The experimental results presented suggest the following final considerations:

1) Thermogravimetric analysis of soot samples collected inside and downstream the spray zone of oil flame allows the calculation of the unreacted fuel and the combustion products, giving a more detailed information on the structure and properties of the spray combustion of liquid hydrocarbons. The same analysis carried out on particulate exhausted from a diesel engine shows cracking products from higher molecular weight materials, the unreacted fuel, the lubricant oil and combustion product at high molecular mass;

2) The comparison of volatile organics associated to particulate obtained by TG and the DCM extractable materials shows that compounds at high molecular mass remain adsorbed in the particulate, both produced in oil flame and in a diesel enigne;

3) The direct sampling in mass spectrometer-data system, by a heated solid probe, of particulates, collected in oil flame spray, gives a detailed information of the highest molecular weight compounds adsorbed on particulate. The same analysis carried out on particulate after DCM extraction can evidence higher compounds, mainly PCAH that remain adsorbed in soot. The compared analysis of soot before and after DCM extraction evidence that the extraction is more incomplete the higher the molecular masses of PCAH were;

4) Direct mass spectrometric analysis of transitional fraction of extracted organic contains PCAH at high molecular masses;

5) Other diesel particulate collection systems, different from the mechanical filtration till now employed, have to be performed in order to obtain sufficient amount of particulate for its complete chemical characterization and health effect evaluation.

ACKNOWLEDGEMENTS

The authors wish to convey their appreciation to Ron Bradow by U.S. EPA-Mobile Source Emission Research for his helpful criticism and Joseph Sturm by U.S. DOT-Transportation System Center for assistance on regulated and unregulated emission measurement. Thanks are also extended to Anna Ciajolo for useful cooperation in experimental work.

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PREPARATION AND CHARACTERIZATION
OF DIESEL EXHAUST PARTICLES
FOR BIOLOGICAL EXPERIMENTS

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ABSTRACT

Bioassays of diesel engine exhaust components are being conducted at IITRI to determine toxic and carcinogenic potentials of the exhaust. The bioassay method, intratracheal instillation of saline suspensions of test materials in hamsters, requires preparation of stable suspensions of test materials.

A method to prepare suspensions of whole particle diesel exhaust in saline has been developed. The diesel exhaust particle material was supplied to IITRI as a dry, loose powder by the U.S. EPA from a light duty diesel test engine. Preliminary characterizations of the powders indicated aggregation of exhaust particles had occurred both before and during capture on collection substrates. Flake-like sheets and hollow spheres of aggregated particles up to 150 μm in size were present in the powders. Therefore, the powders were ball-milled to geometric particle sizes more amenable to the animal administration technique to be employed. Grinding, suspension preparation and particle concentration assaying methods have been developed. Particle (geometric) size and morphological characterizations have also been performed on the as-received powders and prepared suspensions.

A method to prepare emulsions (liquid-liquid suspensions) of the dichloromethane extracts of whole particle diesel exhaust has also been developed.

INTRODUCTION

Under contract to the U.S. EPA Biomedical Research Branch, IIT Research Institute (IITRI) is conducting studies to determine the acute toxicity of diesel engine exhaust components when administered to hamsters by the intratracheal instillation method of Saffiotti (1). The Saffiotti model is designed to determine if the instilled materials possess carcinogenic and/or co-carcinogenic activity relative to the pulmonary epithelium.

Two types of diesel engine exhaust components are being evaluated: whole particle exhaust which is a carbonaceous soot with adsorbed liquid and gaseous species; and a dichloromethane extract of the whole particle exhaust. Both types of materials are being supplied to IITRI by the U.S. EPA Mobile Source Emissions Resources Branch. The methods of exhaust generation, capture and characterization have been described elsewhere (2). Briefly, exhaust generated by a 350 Oldsmobile test engine was diluted and captured on a 20" x 20" teflon-coated glass fiber filter. Continuous operation of the engine resulted in deposition of sufficient solid exhaust material to allow scraping of the exhaust material from the filter. The whole particle diesel exhaust was supplied to IITRI as a loose powder, packaged in glass bottles purged with nitrogen and frozen with dry ice. Extractions of the diesel exhaust-laden filters were performed by the EPA with dichloromethane (3) to prepare the second type of diesel exhaust material to be bioassayed. The extracts were supplied as frozen 86 mg/ml solutions in dichloromethane.

The Saffiotti intratracheal instillation method requires preparation of these two types of diesel exhaust materials as stable suspensions in saline. Gelatin is typically used as a protective colloid for suspension stability purposes. The Saffiotti method also utilizes inert carrier dusts such as ferric oxide (Fe_2O_3), to which the test materials are attached, or at least are intimately mixed with, in order to increase the retention time of the test material at the tissue test site. The bioassay test protocol requires preparation of various concentrations of the two types of diesel engine exhaust materials as gelatin-saline suspensions. This paper describes methods of suspension preparation and characterization.

Whole Particle Diesel Exhaust Suspensions

Microscopical examination of the as-received diesel exhaust powder revealed no significant contamination by teflon and glass fibers from the collection substrate, but that severe agglomeration and aggregation of the submicrometer exhaust particles had occurred. The presence of large diameter (up to 70 μm), hollow carbonaceous spheres indicated some aggregation of the individual exhaust particles had occurred in the exhaust stream, before deposition on the collection filter. The predominant particle morphology--flakes of aggregated particles ranging in thickness from near 0 to 0.5 μm and up to 150 μm in length--could have been formed both before and after deposition on the filter.

To be suitable for intratracheal suspension, the diesel exhaust powder aggregates and agglomerates had to be reduced in geometric size, preferably to a point where 90% by mass of the material was below 10 μm in size. Attempts to disperse the powder into its constituent submicrometer particles by conventional deagglomeration techniques such as ultrasonics confirmed the conclusion that the diesel exhaust particle morphologies observed microscopically were indeed aggregates rather than simple agglomerates. Further microscale testing demonstrated that the only way in which the exhaust particulate material could be dispersed into the individual submicrometer particles was by dissolving the condensed organic species (extractable materials); these sorbed species served as a cementing agent that bound the ultrafine carbon particles in sheets and hollow spheres. Since microscopical analysis indicated that some of the aggregates had been formed before the exhaust was captured on the filter, the most logical approach to reducing the particle size distribution of the powder to a range suitable for intratracheal instillation was to crush the aggregates.

The low density of the diesel exhaust powder, its wide particle size range and its electrostatic nature precluded the possibility of dry grinding the powder before suspension in gelatin-saline. The hydrophobic nature of the powder and sterility requirements suggested that size reduction and suspension in gelatin-saline were best accomplished in one operation.

Before size reduction-suspension methods could be evaluated, a suitable wetting agent for the diesel exhaust powder had to be selected. Although some degree of wetting could be achieved by lengthy, vigorous agitation of very small quantities of the powder with the gelatin-saline, the quantities and concentrations of suspension required for intratracheal instillation ruled out this approach to wetting. Reagent grade propylene glycol was finally chosen as a suitable wetting agent, based on its relatively low toxicity (4), miscibility with saline and non-solubility (at room temperature) of the extractable components of the diesel exhaust.

Low and high intensity ultrasonic treatments, conventional vortex mixers and high speed shear blenders were evaluated as methods of simultaneous exhaust particle size reduction and suspension in gelatin-saline. None of these techniques sufficiently dispersed or reduced sizes of the diesel exhaust particles. A ball-milling technique, developed at IITRI for preparation of benzo(a)pyrene-gelatin-saline suspensions (5), was finally selected as the method of suspension preparation. Milling apparatus consists of wide-mouth, cylindrical pyrex glass jars with silicon rubber-lined plastic caps; 3-5 mm solid pyrex glass beads; and a variable speed roller. Milling vessels containing the appropriate quantities of gelatin and saline were autoclaved before the propylene glycol-wetted diesel exhaust particles were added.

Three concentration ranges of diesel exhaust particle suspensions were each to be prepared with and without equivalent mass concentrations of Fe_2O_3 carrier dust. Table 1 lists the composition of suspensions prepared. Each concentration was prepared individually rather than by dilution of the highest concentration suspension.

TABLE 1. COMPOSITION OF WHOLE PARTICLE DIESEL EXHAUST SUSPENSIONS

Dose Ranges	5, 3 and 1 mg/0.2 ml
Carrier Liquid	Saline with 0.5% w/v gelatin
Wetting Agent	Propylene glycol - 7% by volume
Carrier Dust	Fe_2O_3, 5, 3 and 1 mg/0.2 ml

A milling time of 10 days was required to reduce 75% by mass of the diesel exhaust aggregates below 10 μm. A longer milling time was required to reach the desired size of 90% by mass below 10 μm, but significant contamination of suspensions with glass fragments began to occur after 10 days. Table 2 compares the size distributions of the as-received and milled diesel exhaust particles.

TABLE 2. PARTICLE SIZE DISTRIBUTIONS OF WHOLE PARTICLE DIESEL EXHAUST

Geometric Diameter (μm)	Cumulative Number % Greater Than Size		Cumulative Mass % Greater Than Size	
	As Received	Milled	As Received	Milled
0	100	100	100	100
1.0	83.9	83.7	99.7	99.6
3.0	54.3	48.2	97.2	95.7
5.5	21.9	20.5	82.7	76.1
8.0	10.2	7.9	63.0	48.1
10.5	5.4	2.6	43.6	25.3
13.0	2.3	1.0	26.0	12.4
15.5	1.0	0.3	14.4	4.7
18.0	0.5	0.1	7.3	1.5
20.5	0.2	0	2.5	0

Particles in the suspension quickly agglomerated after milling halted, but could be readily redispersed by simple shaking. However, aged suspensions required more vigorous deagglomeration methods and suspensions stored at room temperature or in a conventional refrigerator for four weeks or more could not be deagglomerated.

Several different types of assays were developed to determine the diesel exhaust particle and Fe_2O_3 concentrations in the final suspensions. Aliquots of each suspension were filtered through tared 0.05 μm pore size membrane filters. This total suspended particle assay provided direct determination of diesel exhaust particle concentration in those suspensions not containing the Fe_2O_3 carrier dust. Diesel exhaust particle concentrations in the suspensions containing the Fe_2O_3 carrier dust were determined by low temperature ashing the filter containing both particle types (from the total suspended particle assay). The material remaining after ashing was the Fe_2O_3 plus a predictable (6) quantity of filter ash. Table 3 presents typical results of the filtration and ashing assays. Reproducibility of the filtration technique was determined to be ±3%. The two different assaying methods produced diesel exhaust particle concentrations within ±5% of each other.

TABLE 3. ASSAY OF 1.0 ml ALIQUOTS OF TYPICAL SUSPENSIONS

	Theoretical Masses (mg)			Assay by Filtration Masses (mg)			Assay by Ashing Masses (mg)		
Suspension*	DP†	Fe_2O_3	Total	DP	Fe_2O_3	Total	DP	Fe_2O_3	Total
DP-Hi	25	-	25	22.8	-	22.8	23.8	-	23.8
DP+Fe-Hi	25	25	50	-	-	50.7	24.4	26.3	50.7
DP-Med	15	-	15	13.9	-	13.9	13.3	-	13.3
DP+Fe-Med	15	15	30	-	-	29.6	13.8	15.8	29.6
DP-Lo	5	-	5	5.3	-	5.3	5.3	-	5.3
DP+Fe-Lo	5	5	10	-	-	9.4	4.4	5.0	9.4

* Hi = 5 mg/0.2 ml
Med = 3 mg/0.2 ml
Lo = 1 mg/0.2 ml

† DP = diesel exhaust particles

Determined diesel exhaust particle concentrations in the prepared suspensions were consistently lower than the theoretical concentrations by 7 to 10%. The low concentrations can be attributed to two factors: solubility of some diesel exhaust components in saline; and the difficulty in completely transferring a particle-liquid suspension from one vessel to another, particularly during separation of the grinding media from the suspension.

Diesel Exhaust Extract Suspensions (Emulsions)

Upon removal of the dichloromethane carrier solvent under a slow stream of nitrogen, the diesel exhaust organic extract was found to be comprised of two phases: a light amber oily liquid; and a semi-solid, dark brown tarry material. Neither material was appreciably soluble in or miscible with saline. Therefore, a wetting agent which, as in the case of the whole exhaust particles, was relatively non-toxic and miscible with saline had to be selected before emulsion preparation could proceed. The two-phase nature of the diesel exhaust extract and the semi-solid nature of the one extract phase required that the wetting liquid essentially dissolved the semi-solid phase. Without solvation, there was no practical method for removing the dichloromethane carrier solvent and transferring the solvent-free extract to an appropriate mixing vessel, or thoroughly mixing the saline with the solid phase in the vessel in which the solvent removal had been carried out.

Since propylene glycol had already been introduced into the bioassay system for the whole diesel exhaust particle suspensions, this was the first solvent tested for use in preparation of the diesel exhaust extract emulsions. At room temperature, the semi-solid phase of the diesel exhaust extract was not particularly soluble in the propylene glycol; however, by gentle heating (50-65°C) the semi-solid diesel exhaust extract phase became more fluid and essentially dissolved in the propylene glycol. The extract oil phase was not miscible with propylene glycol at any temperature.

With propylene glycol selected as a wetting-solvation agent for the diesel exhaust extract, mechanical methods for emulsifying the extract in gelatin-saline were investigated. The need of a surface active agent was immediately evident. Based on conversations with pharmaceutical companies (7) and U.S. Pharmocopeia data (8), sorbitan monooleate--SPAN-80 (9)--was selected as a suitable surfactant. Several different types of conventional mixing emulsifying devices were evaluated for emulsion preparation. Bath-type ultrasonic devices did not provide enough energy to sufficiently disperse the extract components in the gelatin saline, nor did vortex-style mixers. Metal probe-type mixers, including ultrasonic probes and high-speed stirrers, were also found unsuitable because the extract components preferentially adhered to the metal surfaces; no amount of ultrasonic energy would prevent plating out of the diesel exhaust extract on the ultrasonic probe. High-speed blenders with glass blending vessels forced deposition of the extract material on the metal mixing blades, while blenders with metal blending cups resulted in near total loss of the diesel exhaust extract material on both the vessel walls and mixing blades. Teflon coated metal mixer components proved to be no better than the metal mixer surfaces; preferential deposition of significant quantities of extract material on teflon surfaces still occurred. Glass appeared to be the most desirable surface to subject the diesel exhaust extract and gelatin-saline mixture to; no preferential adhesion of extract components occurred. Therefore, glass vessel emulsifying apparatus were evaluated. Standard all-glass Potter-Eljevehm tissue grinders were found to adequately mix and emulsify the diesel exhaust extract in gelatin-saline. Advantages of using such emulsifying apparatus were numerous: mixing apparatus could be sterilized before use; solvent removal from the as-received exhaust extract could be carried out directly in the mixing vessel, thereby eliminating transfer losses; precise weights of extract emulsified with gelatin-saline could be determined; small volumes of emulsion could be prepared, should emulsion stability be a problem; and glass was the only possible contaminant which would be introduced into the system.

Emulsion stability was found to be poor; near complete separation of components occurred with an hour of emulsion preparation. Therefore, an additional stabilizing agent, gum arabic (4), was added to the gelatin-saline before emulsifying with the propylene glycol-diesel exhaust extract mixture. Table 4 lists the components and their concentrations in the final emulsions. Note that all extract emulsions are prepared with Fe_2O_3 carrier dust.

TABLE 4. DIESEL EXHAUST EXTRACT EMULSIONS

Dose Ranges	5, 3 and 1 mg/0.2 ml
Carrier Liquid	Saline with 0.5% w/v gelatin and 0.25% w/v gum arabic
Wetting Agents	Propylene glycol - 10% by volume and Sorbitan Monooleate - 0.1% by volume
Carrier Dust	Fe_2O_3, 5, 3 and 1 mg/0.2 ml

Addition of the gum arabic increased emulsion stability to approximately 12 hours. Although the light oil phase of the diesel exhaust extract separated within minutes of preparation, the oil could be redispersed by simple shaking. The short stability time required that emulsions be prepared fresh for each instillation. Each extract concentration range was prepared individually rather than diluted from the highest concentration emulsion.

At present, no adequate assay method has been developed for the diesel exhaust extract-saline emulsions because of the complex natures of both the extracts and the saline carrier fluid. Assays of the delivered dose of ferric oxide are performed, however. One concern is the possible loss of diesel exhaust extract components during heating with propylene glycol. To minimize this loss, a protective layer of non-heated saline is added before heating is begun.

FUTURE WORK

Although the toxic and carcinogenic potentials of the various non-diesel exhaust components of the suspensions and emulsions being intratracheally instilled are simultaneously tested in the bioassay program (control solvents containing all components except the diesel exhaust material), work is continuing in whole particle diesel exhaust suspension and diesel exhaust extract emulsion preparation methodologies. Other wetting agents, surfactants and protective colloids which are demonstrated to produce suitable suspensions or emulsions are being bioassayed. Other mechanical methods of suspension and emulsion preparation are also being investigated in order to find a mixing device which might allow elimination of some of the wetting, stabilizing and surface active agents.

A simple, routinely applicable assay method for the diesel exhaust extract concentration in the final emulsions is being developed. In all probability, two assay types will be developed: one assay will be occasionally performed to demonstrate that no components of the original diesel exhaust extract have been perferentially lost during storage and handling of the extract; the other assay will be performed on all prepared emulsions to determine diesel exhaust extract concentration by focusing on one or one group of diesel exhaust extract components.

CONCLUSIONS

Methods for preparation of stable suspensions of whole particle diesel exhaust and emulsions of diesel exhaust (organic) extracts in a saline carrier fluid have been developed. Suspensions and emulsions are being intratracheally instilled in hamsters to determine toxic and carcinogenic potentials of diesel exhaust components to pulmonary epithelium.

Methods for assaying concentrations of whole particle diesel exhaust concentrations in the final suspensions have been developed. No methods for assaying the concentration of diesel exhaust extract in prepared emulsions are available; however, methods are being developed.

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SURVEY AND ANALYSIS OF AUTOMOTIVE PARTICULATE SAMPLING

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INTRODUCTION

PARTICULATE EMISSIONS FROM INTERNAL COMBUSTION ENGINES are viewed by the EPA as being potentially harmful to human health, and there is much interest in the accurate sampling and characterization of exhaust particulate. EPA has issued a Recommended Practice to measure the total weight of particulates emitted by a light-duty vehicle over the Federal Test Procedure (FTP). Laboratory measurement techniques follow the general guidelines of the EPA recommended procedure, but there are many parameters that can be varied or are uncontrolled within the scope of the recommended practice. The objective of this paper is to evaluate the effects of those parameters that are identified as having a significant impact on particulate emissions.

The evaluation of these parameters is constrained by the data available and the errors associated with the data. Comparison of data from different sources is sometimes inconclusive because many parameters are varied simultaneously, making it difficult to isolate the effects of each parameter. Thus, the conclusions of the paper reflect the informed judgement of researchers where the data do not provide enough information.

The organization of this paper is as follows: The particulate formation process is briefly described and a general methodology to measure combustion particulate emissions is outlined. This is done to explain the rationale of the EPA Recommended Procedure for particulate sampling from automobiles. The EPA Practice is reviewed and the differences possible in the implementation of this procedure are identified. These differences are grouped into two categories, namely (a) simulation parameters which affect the amount of particulate emitted by the automobile and (b) collection parameters which affect the measured particulate emissions. The effects of parameters in each group are discussed and the error introduced by the parameters on measured particulate emissions are detailed.

PARTICULATE COLLECTION AND RATIONALE

Particulates are defined to be any dispersed matter, both in the solid and liquid phases, present in dilute exhaust gases at conditions close to ambient. The lower limit of particulate size is not defined clearly, but practical considerations and statistical significance place the lower limit at about 100 A°. This value is associated with some representative dimension of the particle, such as the diameter for a spherical particle, or some average dimension for an irregularly shaped particle. Most automotive particulate emissions are composed of many differently sized particulates; size descriptions are usually given in statistical distributions. The size determines the aerodynamic behavior of the particle and this must be accounted for as well in the measurement. The following two sections detail the formation process of exhaust particulates and the rationale for a collection technique that would duplicate this formation process.

Particulate Formation

Researchers have compared the formation of particulates from various combustion sources and found that the size of particulates obtained from natural gas flames, oxyen-acetylene flames, and diesels is similar. Lipkea and Johnson, (1) who have surveyed results of particulate emissions studies from a wide variety of sources, indicate that all combustion produces primary particles that range in size from 0.01 to 10 microns but vary in composition and physical properties, depending on the type of combustion and fuel used. This finding indicates that similar measurement techniques can be applicable to the variety of engines currently used for automotive propulsion such as Otto cycle, stratified charge, and diesel engines.

A simplified diagram of particulate formation in internal combustion engines is given in Figure 1. Khan, _et al_. (2) suggest that the formation of soot particles involves the pyrolysis of fuel hydrocarbons, both in the gas and liquid phase. Nucleation, or the formation of embryonic nuclei,

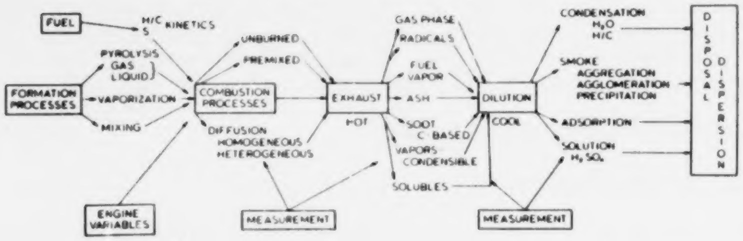

FIGURE 1

PARTICULATE FORMATION PROCESS IN A DIESEL ENGINE

occures in locally fuel-rich areas where combustion is incomplete. This process develops because of the extremely complex turbulent mixing and combustion occuring in the cylinder. Intermediate species are formed in the pre-combustion zones, and these species have side reactions forming polyunsaturated hydrocarbons which lead to the growth of nuclei into soot particles by aggregation. As the exhaust gas passes through the manifold, it is cooled somewhat, and the aggregation processes continue along with the adsorption of hydrocarbons on the surface of the particulate. Physical coagulation or "agglomeration" also takes place and, when the exhaust is released to ambient air, the sudden cooling and dilution causes the hydrocarbon matter to condense, resulting in greater agglomeration and absorption. It is at this diluted level that the particulate matter must be sampled and measured.

Rationale of Measurement Techniques

It is clear that the exhaust pipe and the atmosphere play a part in the formation and growth of particulate matter. Consequently, any laboratory procedure must (a) simulate engine load and speed conditions occurring in normal use, (b) simulate flow through the exhaust pipe and dilute the exhaust emerging from the exhaust pipe with ambient air, and(c) sample the dilute exhaust in such a way that particulates may be trapped without interfering with the particulate growth process or aiding in the formation of artifacts due to measurement. The atmosphere offers the capability of infinite dilution, a situation that cannot be achieved in any simulation. However, several studies have suggested that particulate growth is virtually complete within a few feet of the tailpipe outlet. Dr. Bradow, in an oral presentation to SAE, (3) showed that the dilution ratio remains fairly low (around 10:1 to 20:1) in the immediate vicinity of the tailpipe for an Oldsmobile but rises dramatically (to about 1000:1) as the air vortex from the automobile roof mixes with exhaust. This suggests that laboratory simulation can be realistic at manageable dilution ratios, if particulate formation is complete near the tailpipe as suggested by the studies.

REVIEW OF SAMPLING TECHNIQUES

EPA Recommended Procedure

The procedure recommended by EPA for particulate sampling and measurement is an extension of the basic constant volume sampling (CVS) method used for gaseous pollutant measurement. It must be noted that the procedure only measures the total weight of particulates emitted during the Federal Test Procedure driving cycle. The EPA method involves driving a car on a chassis dynamometer in accordance with the Federal Test Procedure test cycle to simulate typical vehicle speeds and loads. The vehicle tailpipe(s) is connected by a short length of tubing to a diluton tunnel, where the exhaust gas is mixed with filtered ambient air at a dilution ratio that approximates real world conditions. The dilute exhaust is sampled downstream from the point at which air and exhaust are mixed together at a distance sufficient to allow the mixing, hydrocarbon condensation, and agglomeration process to be essentially complete. The sampling is by means of a probe and filter arrangement. The net flow through the tunnel (i.e., the sum of exhaust gas and dilution air) is kept constant, to provide proportional sampling, by means of heat exchanger and a positive displacement pump (PDP) or a critical flow venturi (CFV). EPA's entire sampling system is shown schematically in Figure 2.

Identification of Uncontrolled Parameters

There is wide diversity in the particulate collection schemes employed, and these differences can exist even within the scope of the EPA recommended procedure. The differences, comprised of various uncontrolled parameters, can be divided into two main categories. The first category classified as simulation parameters, includes parameters that simulate the (1) state and heat transfer of the tailpipe; (2) dilution air properties and dilution ratio: (3) effect of mixing air and exhaust to maintain time-temperature-concentration profiles similar to those encountered by the particulate. The second category, classified as collection parameters, includes: (1) the tunnel configuration and the effects of tunnel maintenance procedures; (2) the probe position, type and length of line to the filter; (3) the type of filter or collection apparatus used to trap the particulate. All these uncontrolled parameters can affect the weight, size distribution, organic fraction, and chemical species present on the particulate.

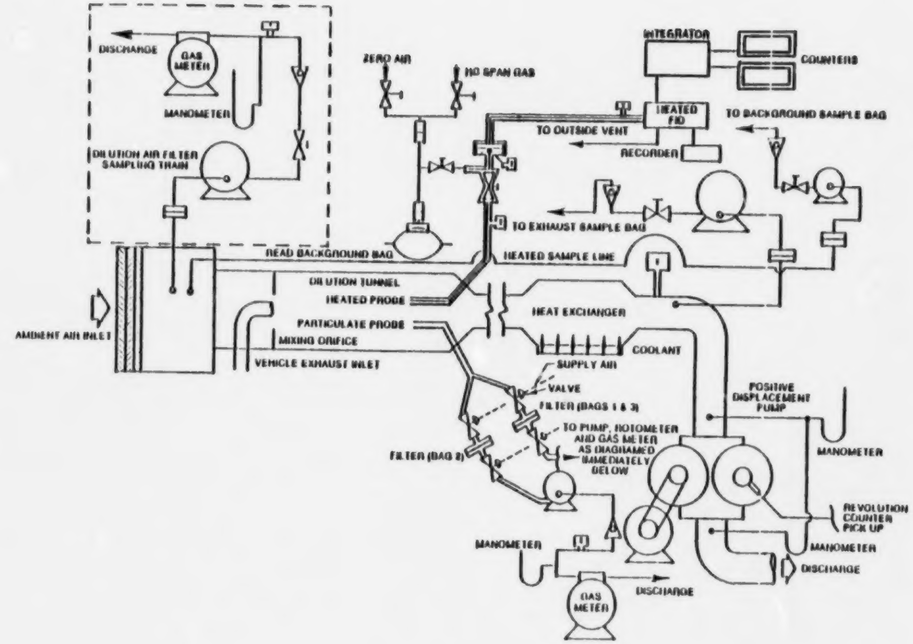

FIGURE 2
SCHEMATIC OF EPA's RECOMMENDED GASEOUS AND PARTICULATE EMISSIONS SAMPLING SYSTEM

EFFECTS OF SIMULATION PARAMETERS

As stated previously, simulation parameters affect the processes that govern particulate formation. This is because, in simulation, the vehicle is stationary on the chassis dynamometer and the exhaust is artificially mixed with a finite amount of dilution air, in contrast to a moving vehicle discharging exhaust into an infinite sink. Because of the interaction between the dilution ratio and mixing rate, these variables are discussed jointly. The effects of specific simulation parameters on particulate formation are detailed below.

(1) Tailpipe State and Heat Transfer Simulation

Theory indicates that the temperature gradients in the tailpipe are sufficiently high to seriously affect the aggregation, agglomeration, and condensation processes occuring in the tailpipe. In addition, the tailpipe has particulate deposits on its walls and produces particulates due to corrosion and wear. The tailpipe and connecting pipe roughness also play a part by trapping particulates on its walls. Simulation of the exhaust pipe state was studied by Danielson, (4) who showed that particulate formation decreases with successive runs at wide open throttle (WOT) (see Figure 3). In the Federal Test Procedure, after a WOT preconditioning, diesel emissions decreased 16 percent in an Oldsmobile diesel but did not decrease in a Mercedes-Benz 300D. This indicates that preconditioning effects are a function of both the exhaust pipe design and the total particulate emissions of the vehicle.

The effect of heat transfer on the exhaust pipe is difficult to examine because airflow under a moving vehicle is difficult to simulate with a stationary vehicle. However, heat transfer effects can be estimated in an indirect fashion by studying the impact of the connecting pipe, which provides additional surface area for heat transfer. In an unpublished study, Danielson (5) found that a long connecting pipe (about 15 ft) with a rough inner surface yielded errors of ±20 percent in particulate emission tests. This error existed when measuring the total weight of particulates; even greater errors are possible when measuring soluble organic matter emissions and individual chemical species. Thus, it appears that heat transfer, exhaust pipe preconditioning, and pipe surface roughness could substantially affect particulate measurement.

(2) Dilution and Mixing of Exhaust

The cooling and dilution of exhaust with filtered ambient air in the dilution tunnel cause some gaseous hydrocarbons to

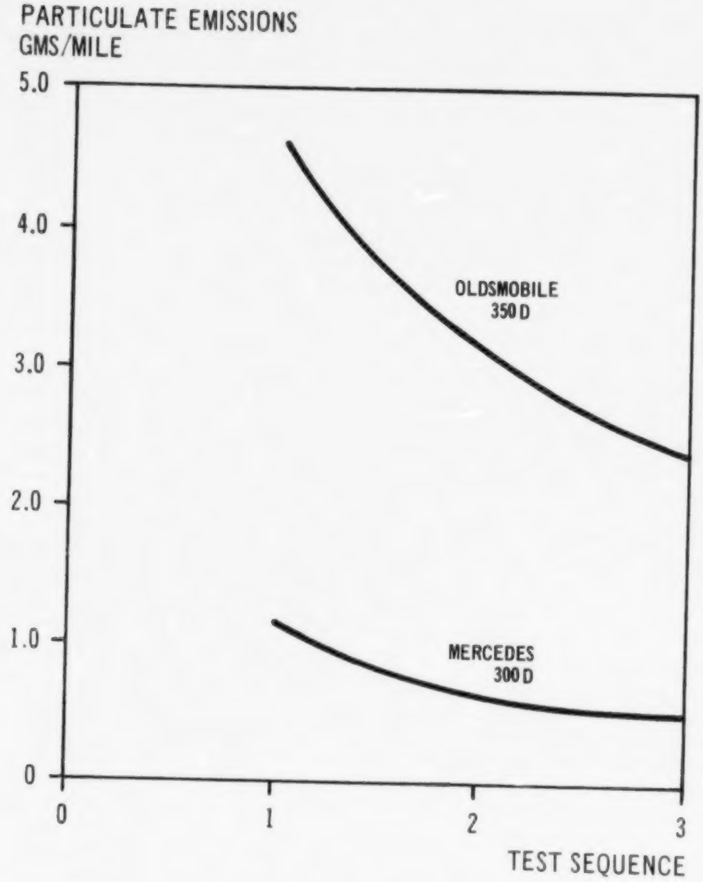

FIGURE 3
PRECONDITIONING STUDY - WOT PARTICULATE EMISSIONS

condense on the particulate surface. Particulate agglomeration and hydrocarbon adsorption/desorption also occur upon cooling and dilution. In the EPA method of measurement, the dilution ratio (or the volumetric ratio of ambient air to exhaust) is not kept constant during particulate emission measurement over the prescribed driving cycle. Rather, the principles of the EPA-specified constant volume sampling method require that the total flow (exhaust plus dilution air) through the tunnel be kept constant and, therefore, the dilution ratio at any instant is inversely proportional to the exhaust flow. The only requirement for dilution is that the peak temperature of dilute exhaust not exceed 125°F.

In the dilution tunnel, the dilution ratio simultaneously determines the final temperature of exhaust, the mixing rate, and the particle residence time in the tunnel. This is because, for a fixed tunnel size, increasing the dilution ratio increases the flow rate through the tunnel which, in turn, increases the rate of mixing of exhaust and air, and decreases the time the particle spends in the tunnel before being sampled. Since the dilution air properties are constant, increasing the dilution ratio lowers the dilute exhaust temperature and also changes the relative humidity of dilute exhaust. Most experiments performed on the effect of dilution ratios do not control for the other variables that are affected simultaneously. The separation of effects of the individual variables is not possible from the data.

Several studies have addressed the effect of the dilution ratio on particulate size distribution. Schreck, et al. (6) measured size distributions of particulate drawn directly from the raw exhaust with a jet pump diluter and from the same exhaust after further transit through the exhaust, 14:1 dilution, and cooling to ambient temperature. They reported that particulate aerodynamic diameter was approximately doubled after transit, dilution, and cooling, in comparison to the former case. Black ahd High (7) measured size distribution at average dilution ratios varying from 8 to 18 and found that particulate size distribution shifted towards the smaller particles with increasing dilution ratios.Laresgoiti, et al. (8) confirmed this result for dilution ratios varying from 4 to 12. Their explanation was that increasing the dilution ratio decreases particle concentration and hence decreases the coagulation rate. The coagulation rate is proportional to the square of the concentration of particles in a given size range, and a lower coagulation rate results in a larger percentage of smaller particles. Due to the differences in the sampling train, the results of Schreck, et al., are not directly comparable to the other results.

The effect of dilution ratio on the weight of particles emitted and the soluble organic content of particulate has been evaluated by several researchers' in comparing the results; errors have been introduced because of the differences in sampling and characterization methods used by the researchers. The differences attributable to the errors associated with the choice of filters or the choice of solvent for extracting the organics may be greater than the variable under study, i.e., the dilution ratio. Laresgoiti, et al. (9) sampled particulate from a Mercedes-Benz OM616 engine, using a glass fiber filter, and found no difference in weight of particulate emissions from raw and dilute (8:1) exhaust. Frisch, Johnson, and Leddy (10) measured particulate emissions from a Caterpillar 3208 heavy-duty diesel engine at various dilution ratios ranging from 0 to 50. They found large increases in total particulate weight with increasing dilution ratios and accounted for this increase in terms of the total soluble organic fraction (DCM* soluble) of the collected particulate. The results, illustrated in Figure 4, also show the effects of mixture temperature and different filter media on this result. Condensation of hydrocarbons due to their high initial concentration may account for these results. Williams and Begeman (11) investigated a light-duty diesel at various steady state speeds with two different dilution ratios. At all speeds except idle, an increase in dilution ratio led to an increase in the percentage of soluble organic matter (solvent: Benzene+EtOH) present on the particulate. The effect of dilution on total weight of particulate was not reported. The results of Laresgoiti, et at., appear inconsistent, but it may be due to the fact that their sampling method did not use a dilution tunnel.

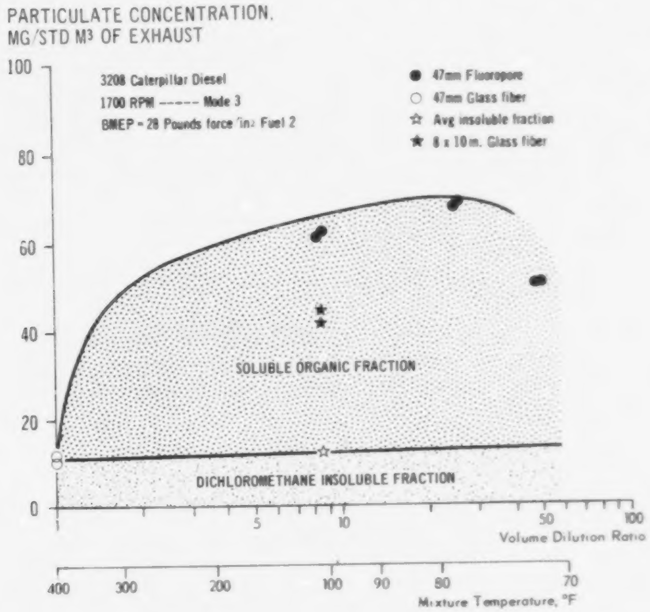

FIGURE 4
EFFECT OF DILUTION RATIO ON PARTICULATE EMISSIONS

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* DCM - Dichloromethane

Black and High (12) performed a series of transient tests on diesel-powered automobiles at different average dilution ratios ranging from 8 to 20. No significant differences were found in the particulate emissions or the soluble organic fraction (DCM soluble) of the particulate. Peak temperature recorded during the tests was 136°F, but the average temperature was below 125°F. Tests by EPA have extended this result to very high dilution ratios, up to 275:1. All dilution values quoted in connection with transient tests are average values, and instantaneous values can differ by as much as an order of magnitude between, for example, the idle mode and high-speed wide-open throttle mode. The results indicate that the dilution ratio is a less important variable for transient tests than for steady-state tests.

Cuthbertson, et al. (13) studied the effect of varying sample temperature alone at constant dilution ratio. The results, illustrated in Figure 5, are based on driving a car over the (hot) FTP and show that particulate weight and the organic content of the particulate are strong functions of filter temperature. Particulate weight reaches a maximum of approximately 125°F. Below 125°F, the results showed considerable scatter, possibly due to the capture of light volatiles on the filter that later are lost or retained, depending on the pre-weighting process. This result indicates that average filter temperatures may have the greatest effect on collected particulate weight. The interactions with the filter are discussed in greater detail in the following section.

EFFECTS OF COLLECTION PARAMETERS

The collection parameters include the apparatus necessary to sample the exhaust and collect the particulate material. The effects of these parameters on particulate emission levels are detailed below.

(1) Tunnel Type and Preconditioning

In spite of a wide variety of tunnel sizes and lengths used in measurement tests, particulate emission results can be affected only if (a) the residence time of exhaust after dilution changes; (b) particulate matter is lost to tunnel walls; or (c) particulate matter and/or hydrocarbons are desorbed from the tunnel wall. Comparisons of emission results from similar automobiles tested in tunnels having different residence times indicate no systematic variations due to tunnel size. Particulate losses to tunnel walls are generally very small. Danielson (14) has shown that successive test results after tunnel cleaning were identical. Tunnel preconditioning and wall losses are, thus, not an important factor in particulate emission measurements.

Nevertheless, researchers recommend that for successive measurements from sources having very different particulate characteristics (such as gasoline and diesel vehicles), the tunnel be cleaned to avoid particulate desorption. Similarly,

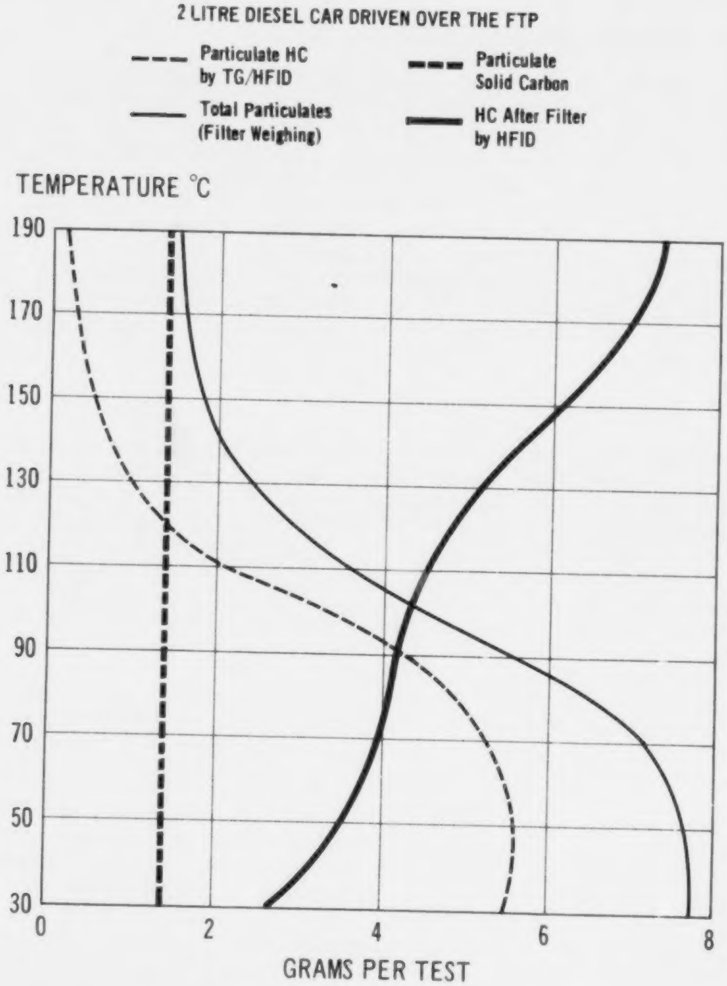

FIGURE 5
VARIATION OF PARTICULATES AND HC WITH FILTER TEMPERATURE

the use of stainless steel walls would preclude desorption of any organic gases or corrosive particles from the wall, although there is no evidence that this is a major factor in measuring particulate emissions. EPA's recommendation for an electrically conductive and grounded tunnel would prevent electrostatic precipitation of particulates due to charged tunnel walls.

(2) Sample Probe and Line Configuration

The effects of the sample probe and line can be subdivided into 1) the need to sample isokinetically and 2) the particulate losses occurring in the line connecting the probe to the filter. There is uniform agreement that, due to the very small particulate size encountered, there is no necessity to sample isokinetically. The effects of isokinetic and non-isokinetic sampling and the effects of sample line length have been investigated by Black and High. (15) Results indicate that emission measurements are not sensitive to probe and line configuration. The effects of probe cleaning and preconditioning also are not expected to affect measurement results.

(3) Trapping Media and Configuration

Although there has been some investigation of electrostatic precipitators, the use of filters to trap particulates is far more common. This later method is useful only for determining total particulate weight and for chemical characterization. Size distribution usually is determined by directly passing dilute exhaust through an optical or mechanical size measuring device. The filter technique can miscalculate factors due to (a) absorption of gaseous hydrocarbons by filter media; (b) artifact formation due to catalysis of chemical reactions by filter media. Filter media normally used are glass fiber, teflon coated glass fiber, and fluoropore membrane filters. Other filters such as quartz fiber and cellulose ester membrane filter also have been investigated.

Black and High (16) measured the sorption of gaseous organics with uncoated glass fiber and Teflon coated glass fiber media. Bench test experiments with gas phase organics alone indicated that the THC loss across the filter was less than 5% for Teflon coated filters versus 25 ±5% of total THC for the glass fiber filters. Tests with vehicle exhaust showed that glass fiber filters (GF/AE) measured consistently higher particulate sample weights than Teflon coated filters; this difference is shown in Table 1. The difference could be accounted for by the difference in organic extracts on the particulate. This appears inconsistent with the results shown in Figure 3, where higher particulate sample weight was

Table 1 - Glass Fiber Versus Teflon Coated Glass Fiber Filter Media (18)

Vehicle	Filter Media	Total Particulate (g/mi)	Particle Extracts (g/mi)	Extracts (% of Total Particulate)
Rabbit	Tef/Glass	0.169	0.046	27.2
Rabbit	GF/AE	0.200	0.072	36.0
Difference		0.031	0.026	
Nissan	Tef/Glass	0.389	0.056	14.4
Nissan	GF/AE	0.408	0.074	18.1
Difference		0.019	0.018	

recorded with Fluoropore filters than with glass fiber filters. In a similar study, Cuthbertson, et al. (17) compared the results of several FTP tests on Diesel cars using Teflon coated (Pallflex) and glass fiber (Watmans type GF/A) filters and found both filters gave identical test results. The reasons for the differences in the results of the three studies are not obvious but may be due to differences between different brands of glass fiber filters.

In a more detailed analysis of artifact formation on the filter, Lee, et al. (18) analyzed various filters with respect to the sample integrity of B(a)P and other PAH compunds present on the particulate. The errors and inefficiency associated with the extraction and dilution of these compounds are reflected in the recovery values for B(a)P and PAH compounds on the filter. The method utilized to study the extent of degradation of B(a)P was the radiotracer method where ^{14}C-B(a)P was used as a model compound and radioactive ^{14}C activity was examined. Table 2 illustrates the percentage recovery of ^{14}C-B(a)P spiked on blank filters after sampling prefiltered ambient air. It is clear that the occurrence of B(a)P/gas reactions depends both on the surface characteristics of the filter and the sample flow rate. It is seen that Tissuequartz and glass fiber filters cause maximum degradation of the sample. It was further shown that the presence of diesel particulate reduced the percentage of ^{14}C-B(a)P recovered from the filter. Many of the PAH in the free molecular state are reactive and, hence, can be expected to undergo facile transformations when exposed to gaseous pollutants. The transformations of ^{14}C-B(a)P to polar and even acidic products were found in the presence of diesel particulate. The dependence of the transformation on the type of filter is high but occurs to some extent on all filters. In the case of Fluoropore filters, the mechanism of ^{14}C-B(a)P loss is not due to air oxidation of B(a)P, nevertheless the mechanism is still unknown.

Attempts to compare particulate obtained by filtration with particulate obtained by other means have led to mixed results. Researchers at GM (19) compared the deposits built up over time on the walls of the dilution tunnel with a fresh filter sample from a diesel vehicle. It was found that the sample from the tunnel walls had a much lower soluble organic fraction than the sample on the filter (9% versus 59.6%), but the solubles were relatively similar in molecular weight distribution. In a similar study by Cuthbertson, et al. (20), fresh deposits from tunnel walls showed exactly the same hydrogen to carbon ratio as the sample on the filter. Since the results obtained by GM were from tunnel deposits that were relatively "old," the prospects of desorption of organics from particulate exists. Tests with stored samples of particulate (21) on the filter have not shown the effects of

Table 2 - The Depencence of B(a)P Recovery on Filter Types and Sampling Conditions (20)

Filter Type	Sampling Conditions[a]					
	Time (hours)	17	8	18	24	30
	Air Volume (m^3)	35	80	140	250	145
	Amount of B(a)P Spiked on the Filter (μg)[b]	0.5	0.5	0.5	0.5	5
Glass Fiber		35	-	-	11	75
Tissuquartz		-	-	1.3	-	62[c]
A Micro Glass Fiber Filter with Teflon Binder on Fibers		-	50	-	40	86
Fluoropore		60	55	65	71	90

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[a] Data listed in the same column represent runs made simultaneously under identical sampling conditions.

[b] The amount of B(a)P spiked on each filter was ~10ng/(cm^2 filter surface) for the first four samples and was ~100ng/cm^2 for the last sample.

[c] This run was made separately but under similar sampling conditions as those runs listed in the same column.

desorption, however. This may be due to the fact that volatile hydrocarbons condense on the filter to form an oily cross-linked structure that is thermally stable, whereas the deposits on the tunnel walls are free from such condensation.

SUMMARY AND CONCLUSIONS

The dilution tunnel and filter method of particulate sampling affect the sample integrity in a number of ways. This paper has examined those parameters that were identified as having affected the weight and character of particulate samples collected; these parameters are dicussed in turn below.

1) The effect of tailpipe heat transfer and preconditioning on particulate emissions is significant. Particulate buildup on the walls is caused by wall roughness and the thermal gradient; the deposited particulate is blown out periodically, especially at non-FTP related driving conditions. The deposited particulate may be higher in organics and nitrated compounds than particulate in the gas stream. Results show that WOT preconditioning can reduce particulate emissions as much as 15% on the FTP. (22)

2) The dilution ratio affects several variables simultaneously, such as the temperature of the mixture of air and exhaust, the rate of mixing, and the residence time of particulate in the tunnel. It is difficult to separate the effects of each of these variables, but it appears that the variable with the strongest effect is the dilute mixture temperature. Most researchers show that collected particulate weight during steady state tests reaches a maximum at temperatures below 125°F. However, it still is not clear whether this result is biased due to the filter sampling technique employed. Transient tests employing variable dilution ratios (and temperature) show particulate emissions to be insensitive to the average dilution ratio employed, as long as the average temperature of the sample was below 125°F; steady-state tests have shown strong effects of varying dilution ratios. This is an indication that the effects of filtration may be masking the effects of dilution ratios on transient tests.

3) Sampling diesel particulate on a filter has been found to lead to a loss of sample integrity due to the continuous sample flow through the filter. Teflon coated filters have been found to be superior to other types of filters to minimize sorption of gaseous organics and catalysis of reactions involving organic compounds, but there are some inconsistent results from glass fiber filters. However, there is no simple method to evaluate the sorption of organics by the particulate matter on the filter. The use of the filtration method leads to the oxidation of PAH compounds and possible reaction of the PAH with pollutant gases such as

NO_x and sulfates, but the extent of sample degradation shows a strong dependence on both sample flow rate and filter type. The particulate sample on the filter also may underestimate the effect of desorption that normally would occur in atmospheric particulate.

Comparisons of a Teflon coated filter and a glass fiber filter measuring particulate emissions show a 15% difference in the total weight of particulate and a 25% difference in the weight of the soluble organic fraction. (23) B(a)P recovery on these filters, using ^{14}C-B(a)P spiked filters that were exposed to a continuous sample of air for 24 hours, was 11% for the glass fiber filter and 71% for the Teflon coated (Fluoropore) filter. (24)

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[a/] Data listed in the same column represent runs made simultaneously under identical sampling conditions.

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[c/] This run was made separately but under similar sampling conditions as those runs listed in the same column.

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19. Schreck, et al., op. cit.

20. Cuthbertson, et al., op. cit.

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24. Lee, et al., op. cit.

General Discussion

R. SCHRECK: Our data is not to be interpreted as saying that increased dilution produces increased particle size. What our results have shown is that you can produce dilution systems that allow for a coagulation effect that will result in a larger particle size.

K. DULEEP: Certainly there are many differences when the entrained sulphur being used is compared by other people. This is just a comparison of data from various sources.

D. KITTLESON: We have done calculations of coagulation in different sampling systems and it can be demonstrated experimentally that an increasing dilution ratio and a decreasing residence time, acting together tends to result in smaller particles. If you go beyond a certain limit however, you freeze out the particle size distribution and there is no further change. The place where care must be taken occurs for example, when running something like a ten to one diluted exhaust, which is typical tunnel dilution, and having residence times much longer than five or ten seconds. Than you can start to get significant shifts. However, if you keep your residence times less than a few seconds under those conditions you shouldn't have any trouble.

K. SPRINGER: I don't believe that the emissions from a diesel engine, the particulates, will be the same from hot filtered raw exhaust as they will be from diluted tunnel exhaust, and I want to take exception to that.

K. DULEEP: I tend to agree with you. This is just a review of all the results and some of the contradictions expressed in the published literature.

A PARTICULATE CHARACTERIZATION STUDY OF IN-USE DIESEL VEHICLES

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Automotive Emissions Laboratory
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ABSTRACT

Particulate sampling methods and vehicle test protocols are described for a study to characterize emissions and fuel economy from approximately 20 diesel vehicles in consumer use throughout a two-year mileage accumulation period. At this time, three diesel cars have been tested by these procedures to collect large quantities of particulate for chemical characterization and bioassay studies to proceed in parallel with testing of in-use vehicles. Primary attention is focused on particle-bound organics removed by solvent extraction of filter samples followed by chemical characterization of extract using GC, HPLC, GC/MS and a new fluorescence mapping technique identified as total luminescence spectroscopy (TLS). TLS results are presented for one particulate extract sample and its sub-fractions to demonstrate the application of this technique.

INTRODUCTION

In anticipation of increased numbers of diesel automobiles in future years, New York State, under EPA support, is conducting a study to obtain characterization data from diesel cars in consumer use. In preparation for repetitive testing of approximately 20 consumer-use diesel cars over a two-year mileage accumulation period, bioassay and analytical tools have been applied to selected samples of diesel particulate, as described in this and other papers at the current symposium (1-3). These analytical and assay methods will be subsequently used to characterize particulate samples from the fleet test portion of the study in which a range of vehicles, fuels, oils, and test conditions will be explored.

Particulate Sampling

The major thrust of characterization effort is aimed at particulate emissions, although regulated emissions and fuel economy are routinely measured by the 1979 Federal Test Procedure for new car certification. Particulate emissions are measured by the standard dilution tunnel technique as detailed in the Federal Register Notice of Proposed Rule Making (4). Some sample-system modifications have been incorporated, as shown in Figure 1. Mass flow controllers have been installed in order to regulate particulate sampling through the 47 mm filters used for determination of emission rate. A system for collecting large particulate samples has been added utilizing a 20" x 20" filter holder. Particulate is collected on the 20" x 20" filter at a nominal 100 cfm and the total volume filtered in any test segment is measured as shown in Figure 1. Pallflex T60A20 Teflon-coated glass fiber filter media are used for both the 47 mm and 20" x 20" filter systems. Exhaust dilution ratio is adjustable by a 10 HP variable-speed motor driving a positive displacement pump CVS. With this drive the CVS flow rate is continuously adjustable from 200-530 cfm, and dilute exhaust temperature is kept below 52°C for different vehicle types and driving cycles.

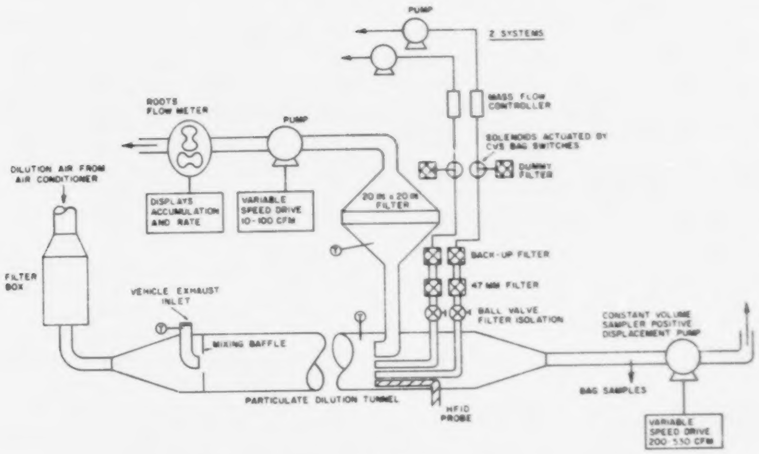
Vehicle Testing

The present testing plan includes three similar one-day phases to complete each vehicle test. These test phases will employ different fuel/oil combinations as follows: 1) fuel as delivered to the Automotive Emissions Laboratory (AEL), oil as delivered to AEL; 2) AEL control fuel, lubricating oil as delivered; and 3) AEL control fuel and fresh lubricating oil. Each day of the vehicle test begins at mid-day with vehicle preparation and an appropriate fuel/oil switch followed by a 30 min 50 mph cruise to purge fuel lines and precondition the vehicle. Immediately following preconditioning, the following cycles are used to determine particulate emission rates and collect large filter samples: 1) a 30 min 50 mph cruise, 2) Congested Freeway Driving Schedule (CFDS), 3) Three successive Highway Fuel Economy Tests (HFET), and 4) a

30 min idle. Gaseous emissions are not measured in this afternoon sequence. After overnight soak, a morning test sequence is begun that includes gaseous/particulate emissions and collection of large filter particulate samples from the following tests: 1) FTP, 2) CFDS, and 3) Three HFET. Fuel and/or oil is then switched to begin the next test phase.

Selected 20" x 20" filters from each three-day vehicle test will be solvent extracted for subsequent chemical characterization and bioassay, with the remaining filters archived in freezer storage for future studies.

Figure 1


PUMP
2 SYSTEMS
MASS FLOW CONTROLLER
ROOTS FLOW METER
PUMP
SOLENOIDS ACTUATED BY CVS BAG SWITCHES
DUMMY FILTER
DILUTION AIR FROM AIR CONDITIONER
DISPLAYS ACCUMULATION AND RATE
VARIABLE SPEED DRIVE 10-100 CFM
20 IN x 20 IN FILTER
BACK-UP FILTER
FILTER BOX
47 MM FILTER
VEHICLE EXHAUST INLET
BALL VALVE FILTER ISOLATION
CONSTANT VOLUME SAMPLER POSITIVE DISPLACEMENT PUMP
MIXING BAFFLE
PARTICULATE DILUTION TUNNEL
HFID PROBE
BAG SAMPLES
VARIABLE SPEED DRIVE 200-530 CFM

SCHEMATIC OF PARTICULATE COLLECTION APPARATUS

Collection of Large Samples

A 20" x 20" filter assembly was used to collect many filters from a few vehicles to provide large samples for detailed studies. A Mercedes 300-D was used to accumulate 180 filters for EPA use in evaluating diesel particulate. The vehicle was operated on a daily schedule that included one FTP for vehicle warm-up followed by 24 HFET cycles broken down into blocks of three cycles to yield one filter from the FTP and eight filters from HFET tests. FTP filters were kept for project analysis and HFET filters shipped to EPA. After completion of this 6,000 mile test program, a similar project of shorter duration was undertaken on the same vehicle using the AEL control fuel scheduled for use throughout the in-use vehicle study.

A diesel Rabbit was subsequently operated with one FTP and 24 HFET cycles each day (AEL control fuel) to generate particulate sample used for detailed characterization analyses described in this and connected papers at the present symposium. A final large sample is now being collected from an Oldsmobile 350 diesel. Frozen portions of extract from all of these large samples will serve as reference bioassay specimens throughout the in-use vehicle portion of the study. Background data on the samples from the Mercedes 300-D and Rabbit are given in Table 1.

Extraction of 20" x 20" Filters

All 20" x 20" Pallflex filters (Type T60A20) used for particle collection were extracted with dichloromethane in order to remove the soluble organic fraction. Filters were folded and placed in 50 mm Soxhlet extractors without thimbles. Extraction was carried out with 300 ml of dichloromethane (Burdick and Jackson Laboratories, Distilled in Glass^{TM} grade) for 24 hr at three to four cycles per hour.

Extracts were filtered by vacuum through an 0.2 μm Fluoropore filter (Millipore FGLP) to remove any particles which may have been carried over. The extract solution was boiled before filtering and the filtering apparatus was kept hot during filtration to prevent precipitation of any material on the filter.

Solvent was removed from the filtered solutions by heat and/or vacuum. When a composite of several filter extracts was being prepared, the volume of each extract was first reduced and the concentrated extracts were combined. Aliquots of the composite were transferred to tared vessels and the remaining

TABLE 1

SUMMARY OF VEHICLE TESTS FOR COLLECTION OF PARTICULATE SAMPLES

	Mercedes 300-D		Rabbit
Fuel	EPA	AEL	AEL
Oil	20W-50, SE-CC	20W-50, SE-CC	20W-50, SE-CC
Particulate Emission(gm/mi) HFET/FTP	0.41/0.63	0.38/0.64	0.35/0.42
Gaseous Emissions, FTP HC/CO/NO_x(gm/mi)	0.22/1.74/1.11	0.22/1.88/1.08	0.31/0.97/0.91
Total Grams Particulate Collected in Large Sample HFET/FTP	255.0/23.1	53.2/4.9	53.8/4.3
Fraction of Particulate Extracted (%) HFET/FTP	10/9	in preparation	29/23
Grams Extract for Characterization Study HFET/FTP	To EPA/2.0	in preparation	15.6/1.0

solvent removed by vacuum at room temperature. These samples were desiccated over Drierite and weighed. Extracted material was fractionated into acidic, basic, and neutral cuts. The neutral fraction was further fractionated into seven cuts on a silica gel column with a gradient of hexane to dichloromethane to ether (2) with partition percentages of 72.9, 6.0, 2.1, 3.8, 4.8, 8.6, and 1.8 for fractions 1 through 7 respectively. Results for the Ames bioassay for these fractions are given in Table 2. Cutpoints for these fractions were adjusted so that Cut 1 contained parafinic material and Cut 2 included the unsubstituted polynuclear aromatic compounds.

Characterization of Organic Extract

In order to characterize organic species adsorbed on carbonaceous particles emitted by diesel engines, a combination of analytical methods and instruments have been employed in a cooperative effort between the Automotive Emission Laboratory of the Department of Environmental Conservation and the Toxicology Laboratory of the Department of Health. The focus of this effort is twofold. The first program area will be the identification of patterns and trends of emissions of adsorbed organic species as a function of engine configuration, fuel, lubricant, and operating conditions. These results are to be used in conjunction with mutagenic activity data in order to relate diesel system operation with possible carcinogenic activity of diesel exhaust. A second task involves use of analytical "fingerprints" of adsorbed organic species in order to infer the possible compounds or classes of compounds which exhibit mutagenic activity. Again, mutagenic activity data is necessary for a systematic determination of the identity of compounds with significant mutagenic impact.

At present, this characterization effort requires four instrument systems. Capillary gas chromatography is used for determination of carbon number distributions (5) of diesel fuel, lubricant, and gross particulate extract. High pressure liquid chromatography is necessary for fractionation of the neutral fraction of the diesel extract. Total luminescence spectroscopy and GC/MS systems are then utilized in order to obtain "fingerprints" of the composition of these extract fractions.

Total Luminescence Spectroscopy

As many different techniques as possible will be employed in an interactive manner in order to explore the many facets of the qualitative and quantitative problems associated with characterization of this material. In this paper, we present some of the capabilities of total luminescent spectroscopy as

TABLE 2

COMPARATIVE AMES TEST RESPONSE (REVERTENTS/μgm SAMPLE)

FOR DIESEL RABBIT PARTICULATE EXTRACT

	TA 98		TA 100	
	-S9	+S9	-S9	+S9
Total Extract	1.4	0.32	3.1	1.0
Subfraction 1	0	0	0	0
" 2	0	0.1	0	0.1
" 3	1.2	1.6	2.3	4.5
" 4	8.3	4.4	16.2	9.0
" 5	1.6	1.4	6.4	7.5
" 6	2.1	1.5	1.6	1.6
" 7	0.6	1.9	0.6	0.2

Details on Fractionation and Ames Test Data given in ref. (2).

an analytical tool for determination of possible compounds and classes of compounds adsorbed on diesel particulate.

Simple luminescence methods have offered sensitivity and specificity for analysis of single compounds. For these methods, use of principle excitation and emission spectrum is often sufficient for identification of a pure compound. However, in mixtures of compounds the overlapping of emission spectra of the individual components often results in the inability to identify peaks corresponding to individual species. Complex mixtures can be analyzed by luminescence techniques only if total luminescence data is acquired and analyzed.

Total luminescence data is the observed intensity of luminescence as a function of all accessible excitation and emission wavelengths. In order to obtain this total picture of luminescence information, we must acquire a large number of emission spectra, each taken for a given excitation wavelength. Contours of equal intensity have been chosen as a convenient method for display of this voluminous data.

Total Luminescence Spectroscopy (TLS) is the term that has been chosen for the computer data acquisition, manipulation, display, and interpretation of total luminescence data. This method has recently (6-8) been found to be an effective tool for identifying crude oil spills, as well as toxic and hazardous materials. Figure 2 illustrates a TLS spectrum for the neutral fraction of extract from a large pooled sample produced by a diesel Rabbit. We note that the upper left region (excitation wavelength > emission wavelength) contains no luminescence information since no emission can occur in this region. Although it is not significant in this case, Rayleigh and Tyndal scattering are centered around the 45° line where the emission wavelength equals the excitation wavelength. Even though there is little structure in Figure 2 because of the superposition of peaks from many individual species, one characteristic group of peaks does appear to be present in the region of 320 to 340 nm excitation wavelengths and 340 to 390 nm emission wavelengths. This structure provides a possible fingerprint of some of the more dominant compounds in a neutral fraction, which probably contains thousands of chemical species. Fractionation of this neutral cut is necessary before more information of the composition of this sample can be obtained.

TLS spectra for this study were obtained on a Baird Corporation SFR-100 Ratio Recording Spectrofluorometer. Data acquisition and scan control for this instrument is locally provided by a Baird MP-100 microprocessor controller which is linked to a host Data General Nova 3 Minicomputer system with 64K words of core and a 10 megabyte disk. Software was

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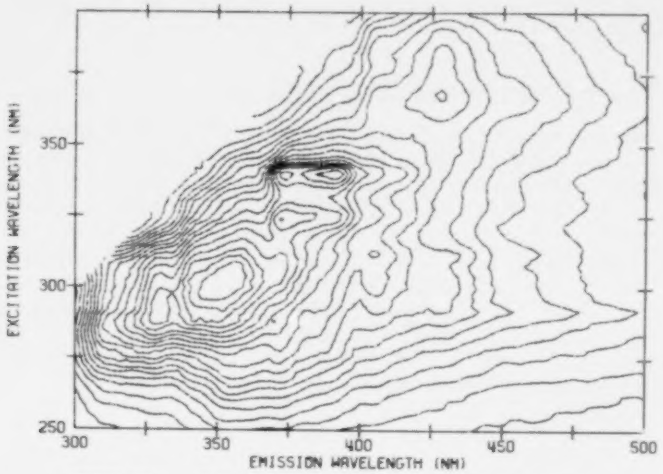
Figure 2



90005-NEU DIESEL RABBIT - NEUTRAL IN DICHLOROMETHANE

MAX.=9.15 X10^{-2} AT 354.0 NM.EM., 304.0 NM.EX.

CONTOUR INTERVAL= 5.00 5.00 TO 95.00 PCT.OF MAX.

supplied by Baird Corporation and modified by AEL personnel with the inclusion of data smoothing algorithems. Contour plots are produced on a Houston Instruments COMPLOT™ X-Y Plotter.

TLS Spectra of Individual Compounds

Some insight into the capabilities of total luminescent spectroscopy can be provided by consideration of spectra of individual pure compounds. Spectra for napthalene, pyrene and fluoranthene in a methylcyclohexane solvent are given in Figures 3-5 in order to illustrate the wide varieties of contour structure found for some polynuclear aromatic compounds. An additional indication of the specificity of the TLS technique is provided by a comparison of spectra obtained for isomers of the same compound. Figures 6 and 7 illustrate the great difference between the contour structure of benzo(a)pyrene and benzo(e)pyrene, respectively.

An additional dimension to the specificity of the TLS method is provided by solvent effects on spectra of the same compound. Compound identification during analysis of mixtures can be aided by taking TLS spectra of the mixture in different solvents. Solvent effects are minimal for some compounds but are significant for other species. These solvent effects can aid in increasing the degree of certainty for compound identification. For example, there is a significant change in the fingerprint for pyrene in dichloromethane (Figure 8) relative to that found for pyrene in methylcyclohexane (Figure 9). By contrast, benzo(a)pyrene does not show any marked shifts in spectra with the same solvents (Figures 10 and 11).

TLS Spectra of Diesel Extract Fractions

Organic extracts of exhaust particulate have been obtained from a 1979 Volkswagon Rabbit Diesel as previously described. Approximately 200 micrograms of each fraction were dissolved in 4 milliliters of solvent (methylcyclohexane, dichloromethane, or methanol) and run on the TLS system. Results for these seven fractions with dichloromethane as the solvent are presented in Figures 12-18.

We note that Cut 1 exhibits a significant fluorescent level. This behavior is expected since the cutpoint on the separation scheme was chosen to include as much of the parafinic material as possible. Therefore, some of the aromatic portion of the sample was included in this parafinic cut.

A complicated contour structure is noted in the Cut 2 spectrum. Identification of compounds by use of the left-hand side of the contour map is difficult because of the severe

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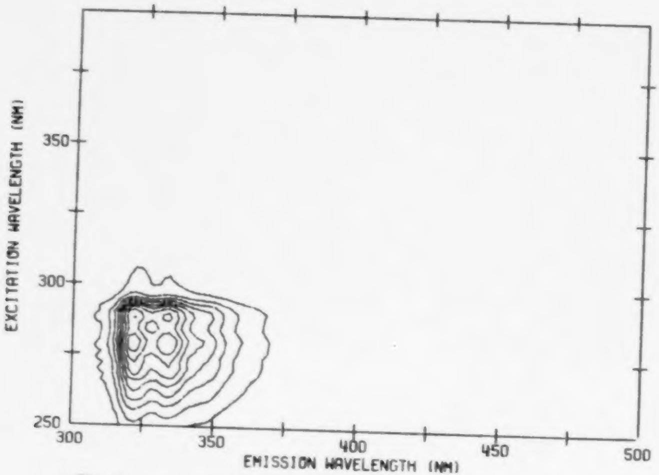
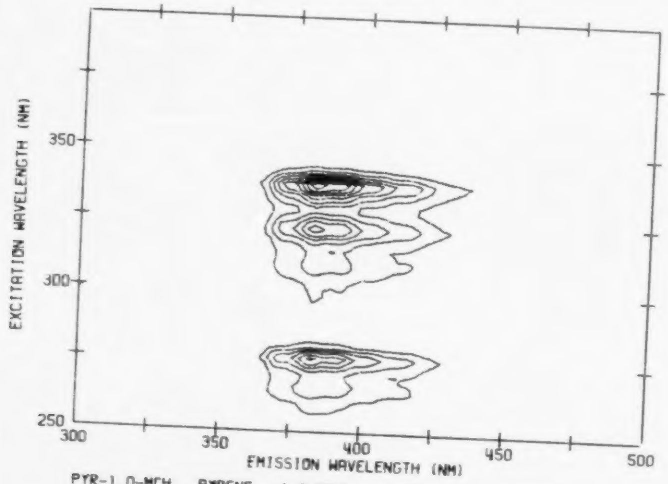
Figure 3



NAP-17.-MCH NAPTHALENE - 17. MG/L IN METHYLCYCLOHEXANE
MAX.=1.40 $X10^{-1}$ AT 322.0 NM.EM.. 278.0 NM.EX.
CONTOUR INTERVAL= 10.00 10.00 TO 90.00 PCT.OF MAX.

11/27/79

Figure 4



PYR-1.0-MCH PYRENE - 1.0 MG/L IN METHYLCYCLOHEXANE
MAX.=4.97 $X10^{-2}$ AT 382.0 NM.EM.. 338.0 NM.EX.
CONTOUR INTERVAL= 10.00 10.00 TO 90.00 PCT.OF MAX.

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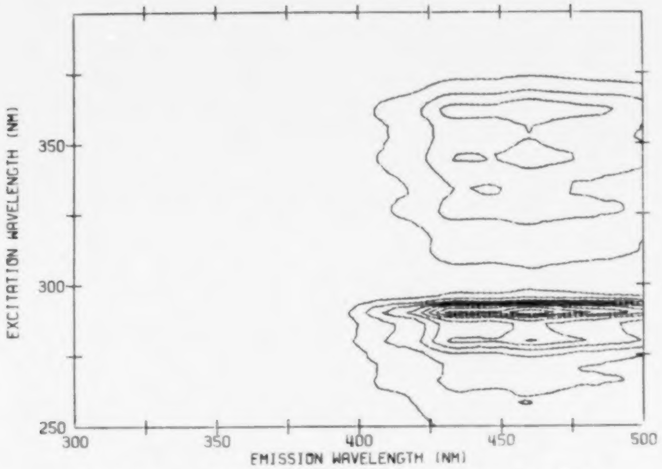
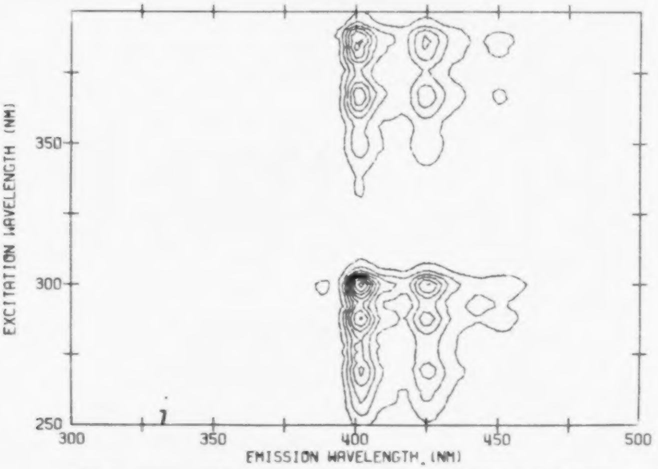
Figure 5



FLU-1.0-MCH FLUORANTHENE - 1.0 MG/L IN METHYLCYCLOHEXANE
MAX.=8.58 X10^{-2} AT 458.0 NM.EM., 290.0 NM.EX.
CONTOUR INTERVAL= 10.00 10.00 TO 90.00 PCT.OF MAX.

11/27/79

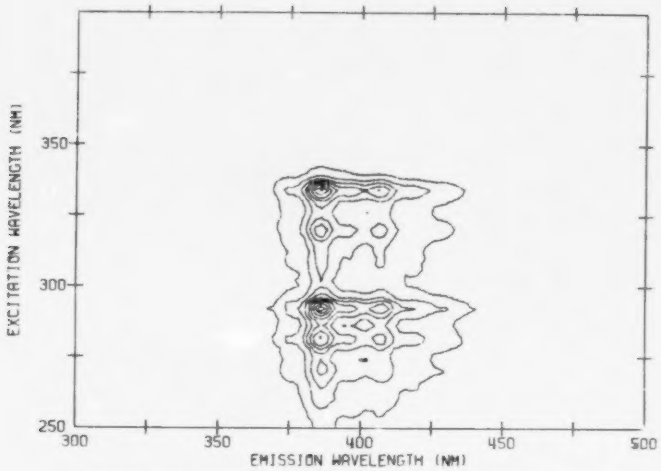
Figure 6



BAP-0.1-MCH BENZO(A)PYRENE - 0.1 MG/L IN METHYLCYCLOHEXANE
MAX.=4.51 X10^{-2}, AT 402.0 NM.EM., 300.0 NM.EX.
CONTOUR INTERVAL= 10.00 10.00 TO 90.00 PCT.OF MAX.

11/26/79

Figure 7



BEP-1.0-MCH BENZO(E)PYRENE - 1.0 MG/L IN METHYLCYCLOHEXANE
MAX.=4.65 X10^{-2} AT 386.0 NM.EM., 292.0 NM.EX.
CONTOUR INTERVAL= 10.00 10.00 TO 90.00 PCT.OF MAX.

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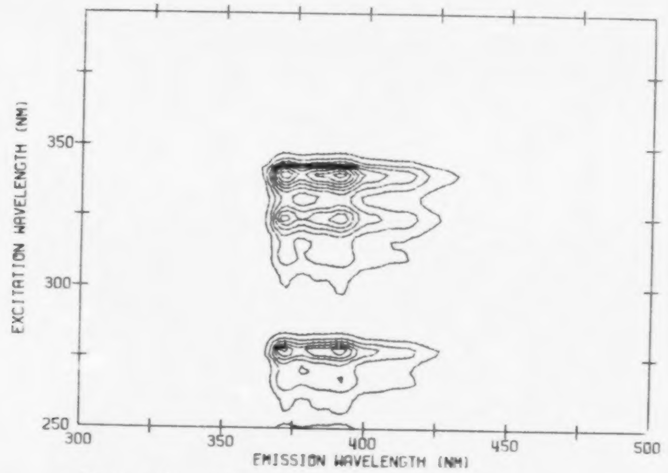
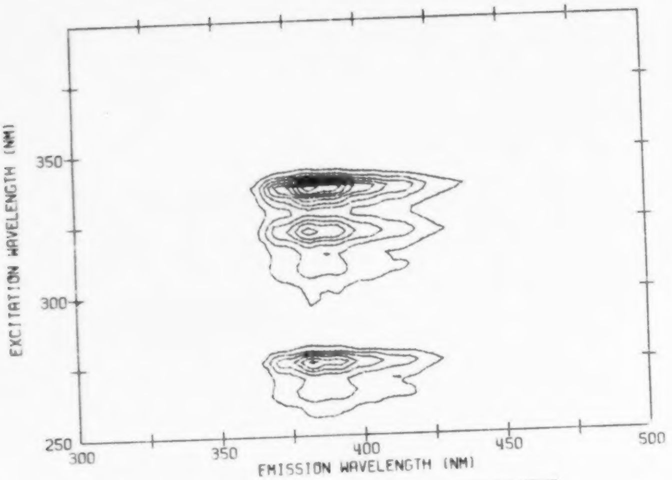
Figure 8



PYR-1.0-DCM PYRENE - 1.0 MG/L IN DICHLOROMETHANE
MAX.=1.39 X10^{-1} AT 390.0 NM.EM., 340.0 NM.EX.
CONTOUR INTERVAL= 10.00 10.00 TO 90.00 PCT.OF MAX.

11/27/79

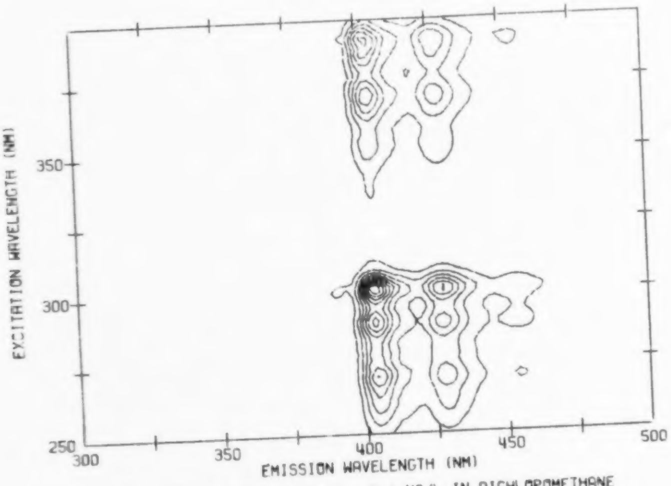
Figure 9



PYR-1.0-MCH PYRENE - 1.0 MG/L IN METHYLCYCLOHEXANE
MAX.=4.97 X10^{-2} AT 382.0 NM.EM., 338.0 NM.EX.
CONTOUR INTERVAL= 10.00 10.00 TO 90.00 PCT.OF MAX.

11/27/79

Figure 10



BAP-0.1-DCM BENZO(A)PYRENE - 0.1 MG/L IN DICHLOROMETHANE
MAX.=8.13 X10^{-2} AT 404.0 NM.EM., 302.0 NM.EX.
CONTOUR INTERVAL= 10.00 10.00 TO 90.00 PCT.OF MAX.

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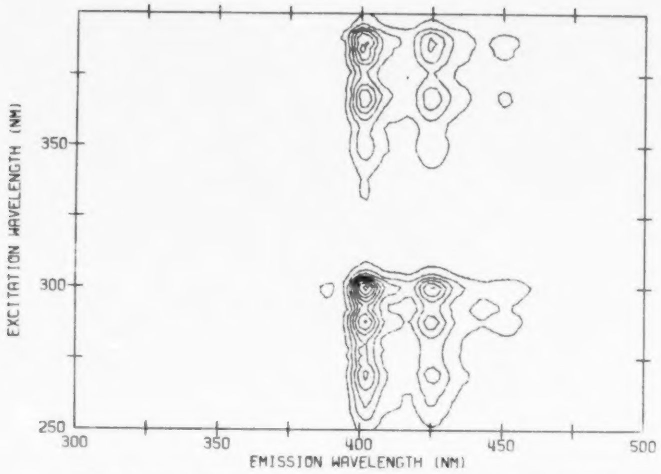
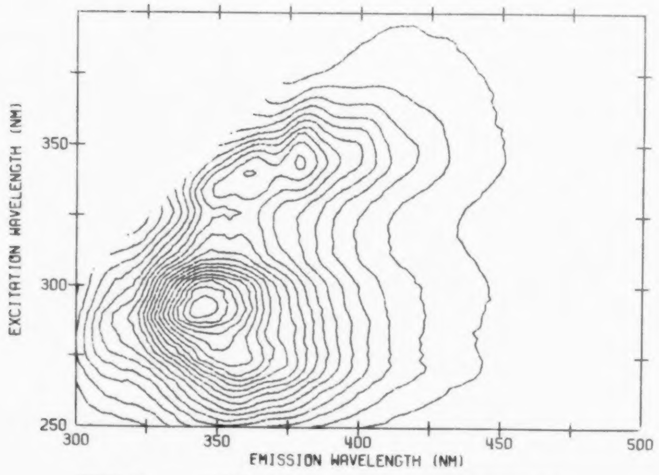
Figure 11



BAP-0.1-MCH BENZO(A)PYRENE - 0.1 MG/L IN METHYLCYCLOHEXANE
MAX.=4.51 X10^{-2} AT 402.0 NM.EM., 300.0 NM.EX.
CONTOUR INTERVAL= 10.00 10.00 TO 90.00 PCT.OF MAX.

11/26/79

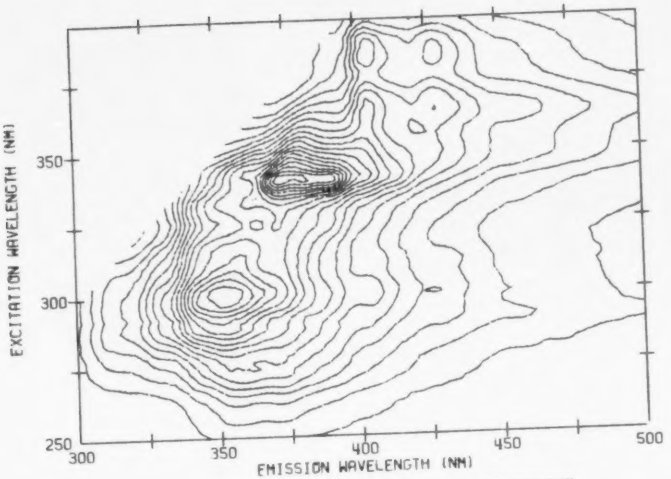
Figure 12



90005-C1 DIESEL RABBIT - CUT 1 IN DICHLOROMETHANE
MAX.=1.64 X10^{-1} AT 344.0 NM.EM., 292.0 NM.EX.
CONTOUR INTERVAL= 5.00 5.00 TO 95.00 PCT.OF MAX.

11/21/79

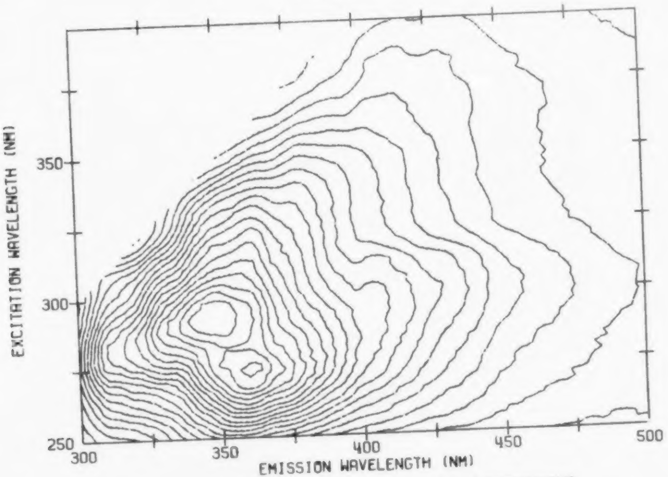
Figure 13



90005-C2 DIESEL RABBIT - CUT 2 IN DICHLOROMETHANE
MAX.=1.82 X10^{-1} AT 372.0 NM.EM., 340.0 NM.EX.
CONTOUR INTERVAL= 5.00 5.00 TO 95.00 PCT.OF MAX.

11/26/79

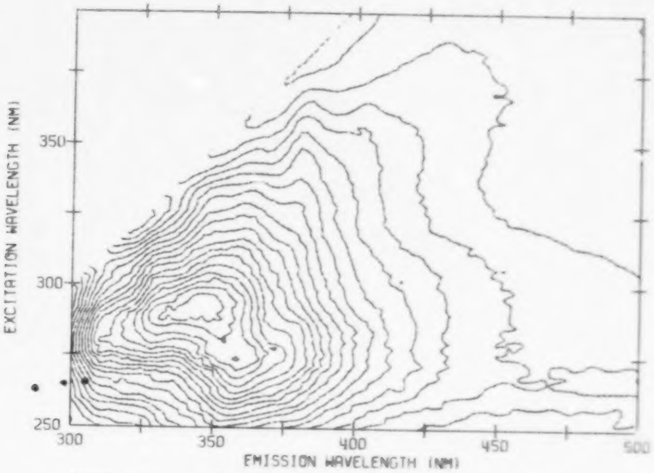
Figure 14



90005-C3 DIESEL RABBIT - CUT 3 IN DICHLOROMETHANE
MAX.=1.53 X10^{-1} AT 352.0 NM.EM., 292.0 NM.EX.
CONTOUR INTERVAL= 5.00 5.00 TO 95.00 PCT.OF MAX.

11/26/79

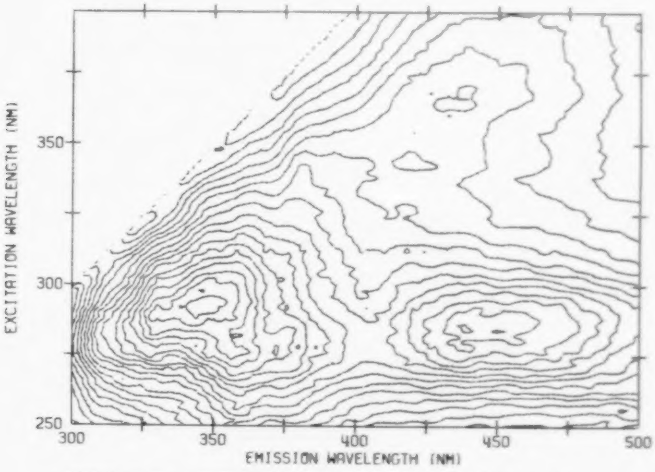
Figure 15



90005-C4-M DIESEL RABBIT - CUT 4 IN DICHLOROMETHANE
MAX.=4.55 X10^{-2} AT 348.0 NM.EM., 296.0 NM.EX.
CONTOUR INTERVAL= 5.00 5.00 TO 95.00 PCT.OF MAX.

11/26/79

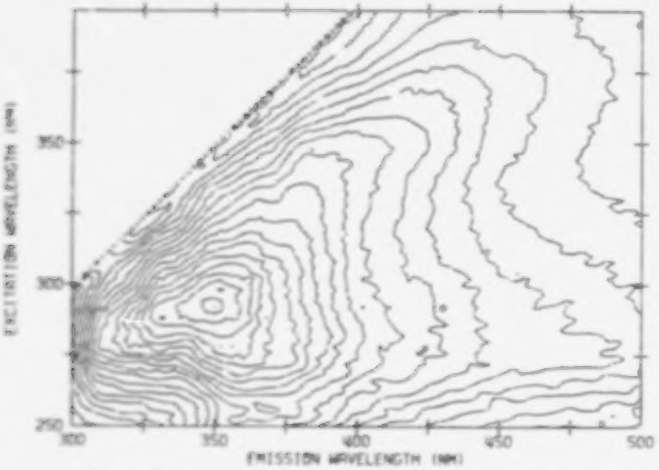
Figure 16



90005-C5-M DIESEL RABBIT - CUT 5 IN DICHLOROMETHANE
MAX.=2.49 X10^{-2} AT 350.0 NM.EM., 294.0 NM.EX.
CONTOUR INTERVAL= 5.00 5.00 TO 95.00 PCT.OF MAX.

11/29/79

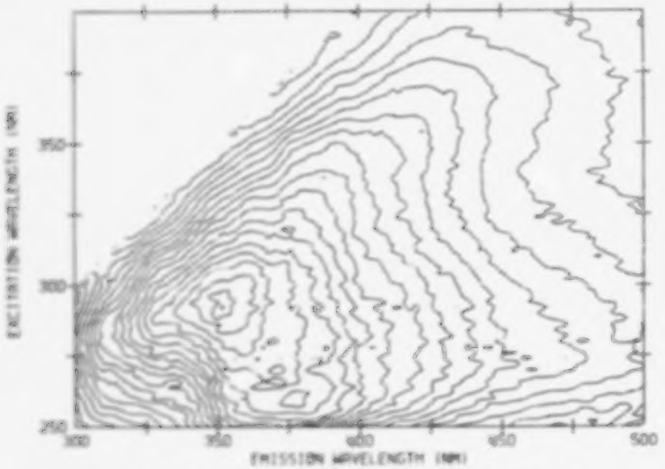
Figure 17



90005-C6-M DIESEL RABBIT - CUT 6 IN DICHLOROMETHANE
MAX.=2.97 X10^{-6} AT 348.0 NM.EM. - 294.0 NM.EX.
CONTOUR INTERVAL= 5.00 5.00 TO 95.00 PCT.OF MAX.

11/29/79

Figure 18



90005-C7-M DIESEL RABBIT - CUT 7 IN DICHLOROMETHANE
MAX.=2.20 X10^{-6} AT 352.0 NM.EM. - 280.0 NM.EX.
CONTOUR INTERVAL= 5.00 5.00 TO 95.00 PCT.OF MAX.

overlap of spectra in this region. However, the characteristic pattern of benzo(a)pyrene (see Figure 6) is noted in the upper portion of Figure 13. This benzo(a)pyrene fingerprint is clearer in Figures 19a and 19b where the same information as Figure 13 is presented for methylcyclohexane solvent. In addition, the lower portion of this spectrum is presented with a fine contour grid. The characteristic ridges of fluoranthene at 290 and 360 nm (see Figure 5) are also found in Figure 19b. Cut 2 was also analyzed by TLS in dichloromethane as shown in Figure 20. Although the benzo(a)pyrene and fluoranthene structure is weaker with this solvent, the pyrene (see Figure 8) spectra is more evident in Figure 20 than in Figure 19a.

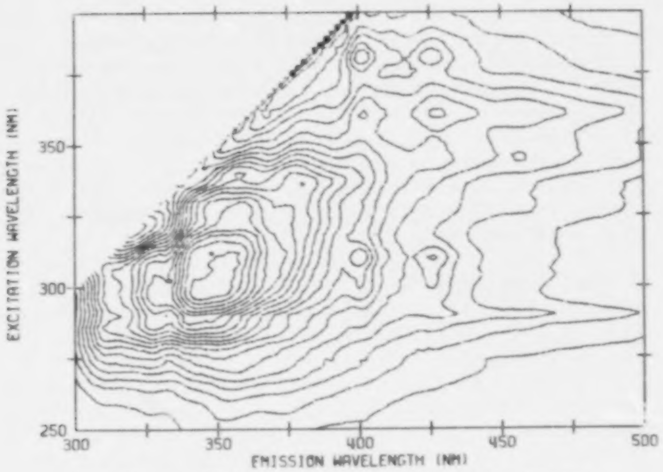
Another manifestation of the use of solvent effects in the compound identification process is illustrated by the contour maps for Cut 5 in methylcyclohexane (Figure 21) and in dichloromethane (Figure 22). A low overall level of fluorescence for this fraction results in noisy spectra, although the smoothed results of Figures 21 and 22 yield a reasonable amount of contour detail. Although the broad peak in the area of 285 nm excitation, 450 nm emission is visible in the methylcyclohexane spectrum, a much clearer picture of this fluorescent contour structure is found in the TLS spectrum of Cut 5 in dichloromethane.

Quantitative information can also be obtained from TLS. Available software enables the plotting of any excitation or emission scan. For example, the principle excitation and emission scans for benzo(a)pyrene in methylcyclohexane are presented in Figures 23 and 24 respectively. Analysis of TLS results for the 386 nm excitation scan of Cut 2 in methylcyclohexane coupled with the TLS spectra of pure benzo(a)pyrene indicates that benzo(a)pyrene accounts for 0.2 weight percent of Cut 2, which translates to an effective emission rate for benzo(a)pyrene of 9.0 micrograms per mile.

Before serious extract characterization and compound identification can be initiated, a library of TLS spectra for pertinent pure compounds must be produced. The software library must be modified to incorporate a soon-to-be-released addition-subtraction program. This program will allow subtraction of known compounds from the spectra in order to produce a clearer picture of the remaining contour structure. In addition, the addition-subtraction routine will allow the determination of quantitative information for extract fractions which contain small numbers of fluorescing compounds.

11/21/79

Figure 19a



9005-C2-M DIESEL RABBIT - CUT 2 IN METHYLCYCLOHEXANE
MAX.=1.84 X10^{-1} AT 348.0 NM.EM., 300.0 NM.EX.
CONTOUR INTERVAL= 5.00 5.00 TO 95.00 PCT.OF MAX.

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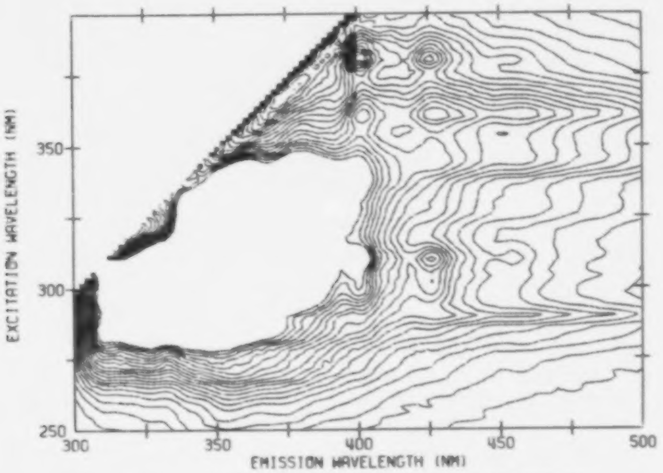
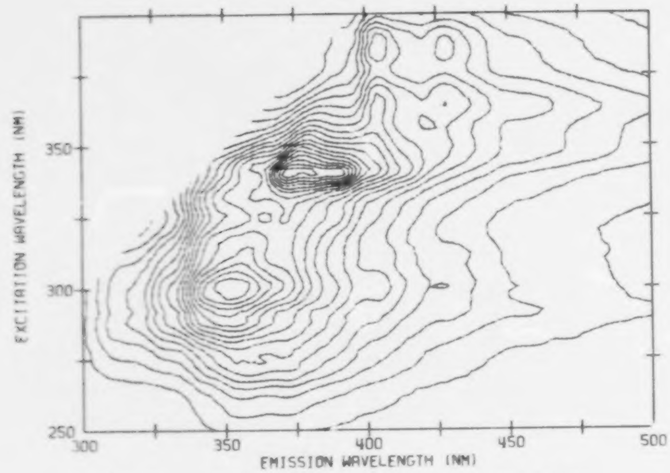
Figure 19b



9005-C2-M DIESEL RABBIT - CUT 2 IN METHYLCYCLOHEXANE
MAX.=1.84 X10^{-1} AT 348.0 NM.EM., 300.0 NM.EX.
CONTOUR INTERVAL= 2.00 2.00 TO 40.00 PCT.OF MAX.

11/21/79

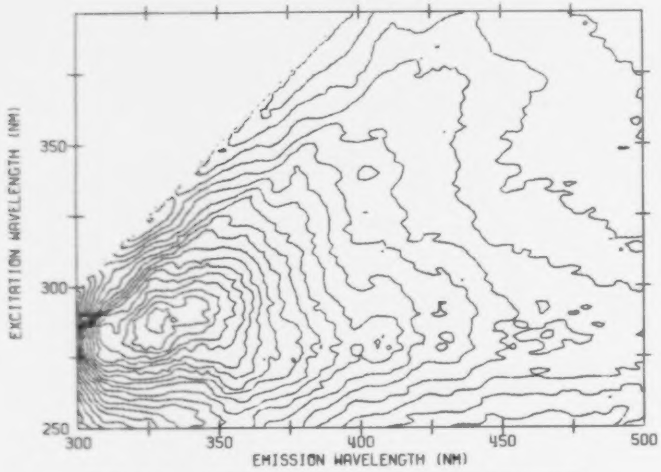
Figure 20



90005-C2 DIESEL RABBIT - CUT 2 IN DICHLOROMETHANE
MAX.=1.82 X10^{-1} AT 372.0 NM.EM., 340.0 NM.EX.
CONTOUR INTERVAL= 5.00 5.00 TO 95.00 PCT.OF MAX.

11/26/79

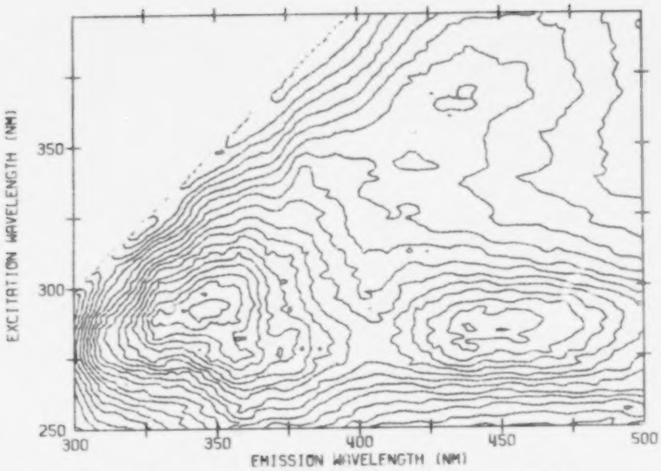
Figure 21



90005-C5-M DIESEL RABBIT - CUT 5 IN METHYLCYCLOHEXANE
MAX.=2.23 X10^{-2} AT 328.0 NM.EM., 288.0 NM.EX.
CONTOUR INTERVAL= 5.00 5.00 TO 95.00 PCT.OF MAX.

11/26/79

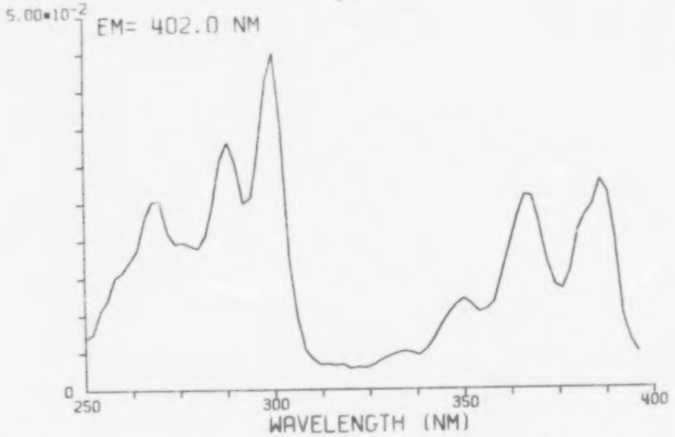
Figure 22



90005-CS-M DIESEL RABBIT - CUT 5 IN DICHLOROMETHANE
MAX.=2.49 $X10^{-2}$ AT 350.0 NM.EM., 294.0 NM.EX.
CONTOUR INTERVAL= 5.00 5.00 TO 95.00 PCT.OF MAX.

11/26/79

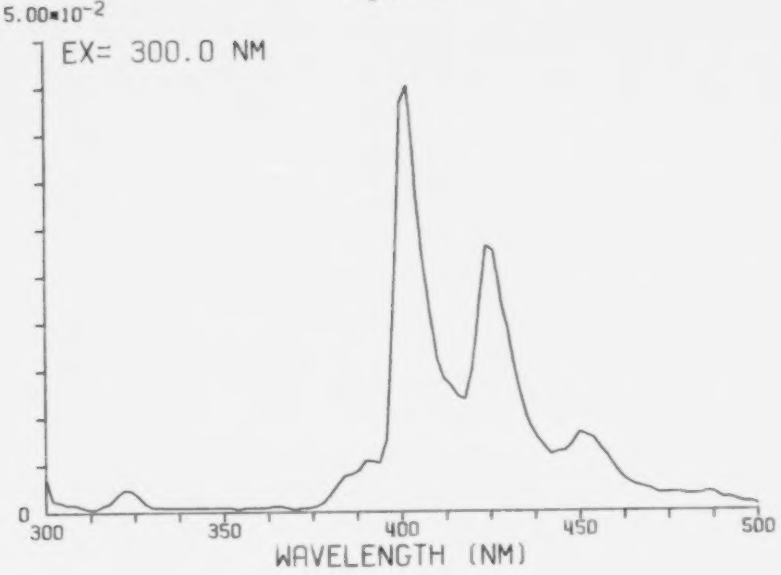
Figure 23



BAP-0.1-MCH BENZO(A)PYRENE - 0.1 MG/L
IN METHYLCYCLOHEXANE

11/26/79

Figure 24



BAP-0.1-MCH BENZO(A)PYRENE - 0.1 MG/L
IN METHYLCYCLOHEXANE

Acknowledgements

Appreciation is directed to Stan Byer in helping with the arduous procurements necessary for this project, Ben Hill for help in the computer software developments, and Bob Johnson and Paul Werner in performing the vehicle testing. This work was partially supported through EPA research grant R805934010.

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General Discussion

P. THILLY: Maybe I forget my LCAO modeling, but it seems to me that in a system such as diesel soot overloaded with methyls, dimethyls and ethyl substituents, that this procedure would more or less lump such substituent compounds and not shift their behavior in your system. Yet such substitutions would be important from a first chemical characterization and secondly from a biological characterization. Am I in error in remembering that result?

G. WOTZAK: What you find with simple methyl and ethyl substitution, there isn't any great shifts in the TLS spectra involved. What you do find when you have substitution of, let's say, CHOH group or NH2 or something like this, is that there are significant solvent effects in terms of shifting over to the right and upward of the whole spectra involved. You tend to lump some of the simple alcohol substitutions involved for the PAH's involved here. That is one problem you have in this, but you can get some fallout of any of the oxygenated compounds. Just from what we have seen here you have the whole thing with rather simple PAH's and also the oxygenated species. You are going to be able to see some differences both from just plain TLS spectra plus also going to high polarity solvents. If you go from methyl cyclohexane to dichloro methane, going up to methanol, higher polarity, you notice some shifts of some oxygenated species. So there is another case where solvent effects may get a picture of what type of compounds is involved.

MEASUREMENT OF UNREGULATED EMISSIONS - SOME HEAVY DUTY DIESEL ENGINE RESULTS

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Caterpillar Tractor Co.
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ABSTRACT

Development of analytical capabilities to evaluate unregulated emissions are discussed. The sampling and analysis methods along with some problem areas are included. Results obtained on three heavy-duty diesel engines are reported. Emphasis was placed on analysis of the particulate fraction including the solvent extractable material. Preliminary experiments suggest appreciable quantities of organic extract and BaP pass through the primary filter. Other species analyzed to obtain a baseline for emission reduction research included aldehydes, sulfates, sulfur dioxide, ammonia, hydrogen sulfide and hydrogen cyanide. Of primary concern is the proper assessment of the results.

INTRODUCTION

The composition of diesel exhaust has challenged investigators with its complexity. Studies in the late sixties and early seventies, stimulated by an interest in exhaust reactivity or diesel odor, did produce some information on diesel exhaust composition (1,2,3,4).*

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*Numbers in () are References at end of text.

However, most research on diesel emissions was aimed at measuring and reducing gaseous emissions (5,6,7,8). The projected increase in diesels (9) and the issuance of Advisory Circular-76 coupled with recent advances in chromatography and spectroscopy have resulted in increased efforts to chemically characterize and measure diesel exhaust constituents. This is evidenced by recent publications on both light (10) and heavy duty diesel (11,12) exhaust composition.

This paper discusses some of our current efforts to measure the constitutents shown in Table 1 and to characterize diesel exhaust. Caterpillar Tractor Co.'s research program on the chemical composition of diesel exhaust dates back some 30 years. Early work by Dr. E. W. Landen on smoke, nitric oxide and fuel composition effects (13,14) demonstrated the advantages of physical and chemical diagnostics on hardware development and resulted in a continuous program in this area.

Upon receipt of Advisory Circular-76 the decision was made to build-up our in-house capability to measure unregulated emissions. This required increasing our commitment in facilities and people in this area as well as addition of new equipment. Selection of methods and instrumentation was facilitated by visits to various laboratories working in the field. The cooperation of laboratories such as Southwest Research Institute (SwRI), Department of Energy (DOE) Bartlesville and EPA-RTP were very helpful.

Use of an outside contract laboratory, Illinois Institute of Technology Research Institute (IITRI), helped to accelerate some method developments during the build-up phase of the program. Personnel also attended instrument suppliers workshops. These efforts resulted in tested methodology and trained personnel and within 6 months measurement of unregulated emissions was started. Our laboratory facility, Figure 1, has limited access, special lighting and adequate temperature and humidity control.

ANALYTICAL PROCEDURES

SAMPLING

The sampling system used in this work is shown in Figures 2A and B. The mini-dilution tunnel shown in Figures 2A and B is a 17.8 cm x 3.1 meter tunnel of 500 cfm capacity. Dilution air enters through an Ultra Aire^R filter and is mixed with raw exhaust by a converging-diverging nozzle and orifice plate. The raw exhaust flow is controlled by a

TABLE 1 UNREGULATED EMISSIONS

ALDEHYDES
AMMONIA
HYDROGEN CYANIDE
HYDROGEN SULFIDE
METALS
NITROSAMINES
POLYNUCLEAR AROMATIC HYDROCARBONS
    BENZO – α – PYRENE
    BENZO – α – ANTHRACENE
SOLVENT EXTRACTABLE FRACTION OF PARTICULATES
TOTAL PARTICULATES
SOLUBLE SULFATE
SULFUR DIOXIDE

FIGURE 1 UNREGULATED EMISSIONS LABORATORY

FIGURE 2a CATERPILLAR MINI-DILUTION TUNNEL

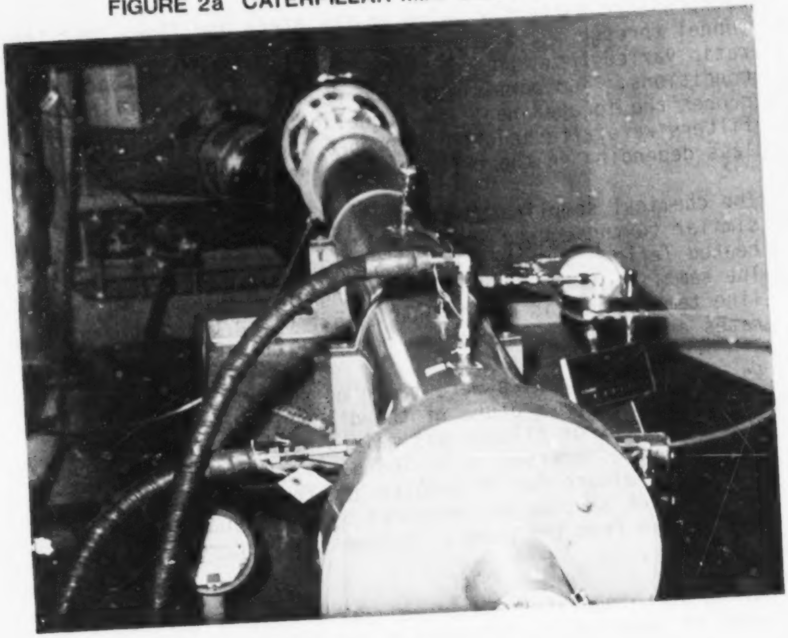
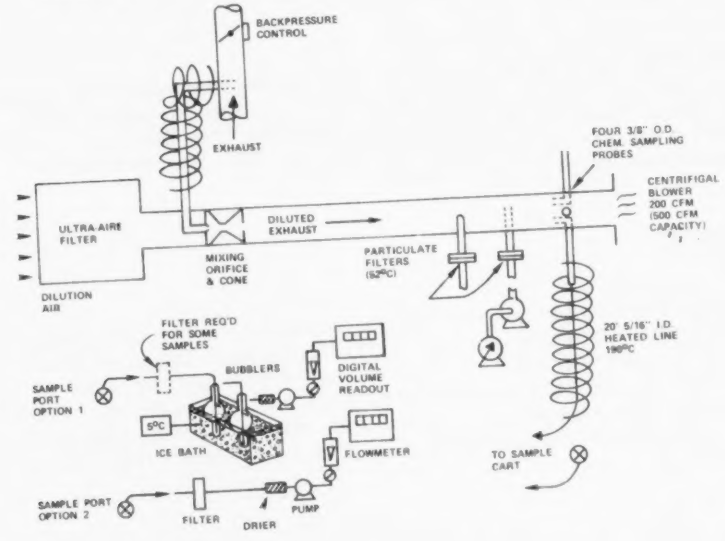


FIGURE 2b UNREGULATED EMISSIONS SAMPLING SYSTEM



backpressure butterfly valve in the stack and flows into the tunnel through a 1.5 meter, 0.95 cm O.D. line. Dilution ratio varied from about 10:1 to 15:1 depending on operating conditions. Two particulate and four chemical sampling probes are located near the tunnel outlet. The particulate filters were attached to the probes and held at 52° C or less depending on the exhaust flow into the tunnel.

The chemical sampling carts shown in Figures 3A and B are similar to those used at SwRI (12). Each cart has two heated Teflon^R sampling lines and four sampling stations. The sampling lines are 6 meters long and 0.95 cm I.D. The line temperature is maintained at 52° C and the sampling rates are varied depending on the species sampled.

In general the samples were taken as recommended by Reference 15. The temperature of the diluted exhaust to the glass bubblers or filters was maintained at 52° C. The bubblers were immersed in an ice bath. The flow rate was 4 liters per minute for 30 minutes and the volume of sample through each sampler was measured by individual gas meters down-stream from the pumps. Volumes were corrected to STP conditions.

During an unregulated emissions engine test, Figure 4, a total of some 175 to 200 samples are collected over a five-day period for subsequent chemical analysis.

CHEMICAL METHODS

The chemical speices analyzed and the methods used are shown in Table 2 and are described briefly in the following paragraphs. Some of the methods are adopted from Reference 15 which details procedures validated by SwRI for unregulated emissions in gasoline exhaust.

Ammonia

Ammonia in diesel exhaust can be measured in the protonated form NH_4^+, after collection in dilute H_2SO_4. The acidification is carried out in two glass bublers in series maintained at ice bath temperature, each containing 25 mL 0.01 N H_2SO_4. The sample is analyzed for ammonium ion in the ion chromatograph and compared to standards of known NH_4^+ concentrations.

FIGURE 3a CATERPILLAR SAMPLING CART

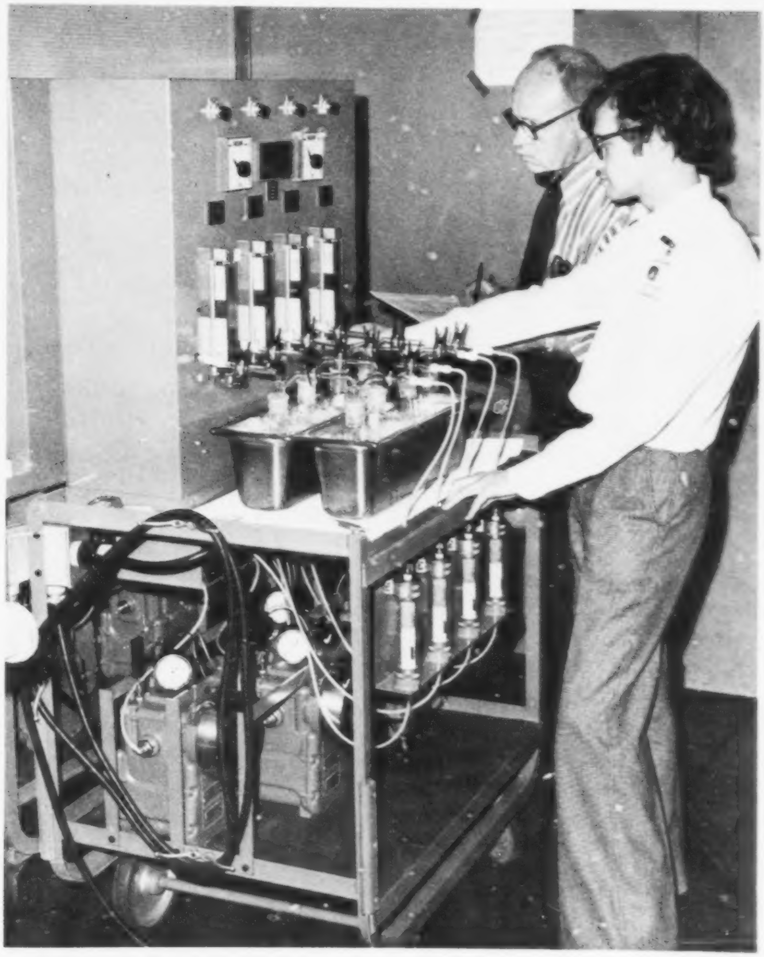


FIGURE 39: SAMPLING CART (SCHEMATIC DIAGRAM)

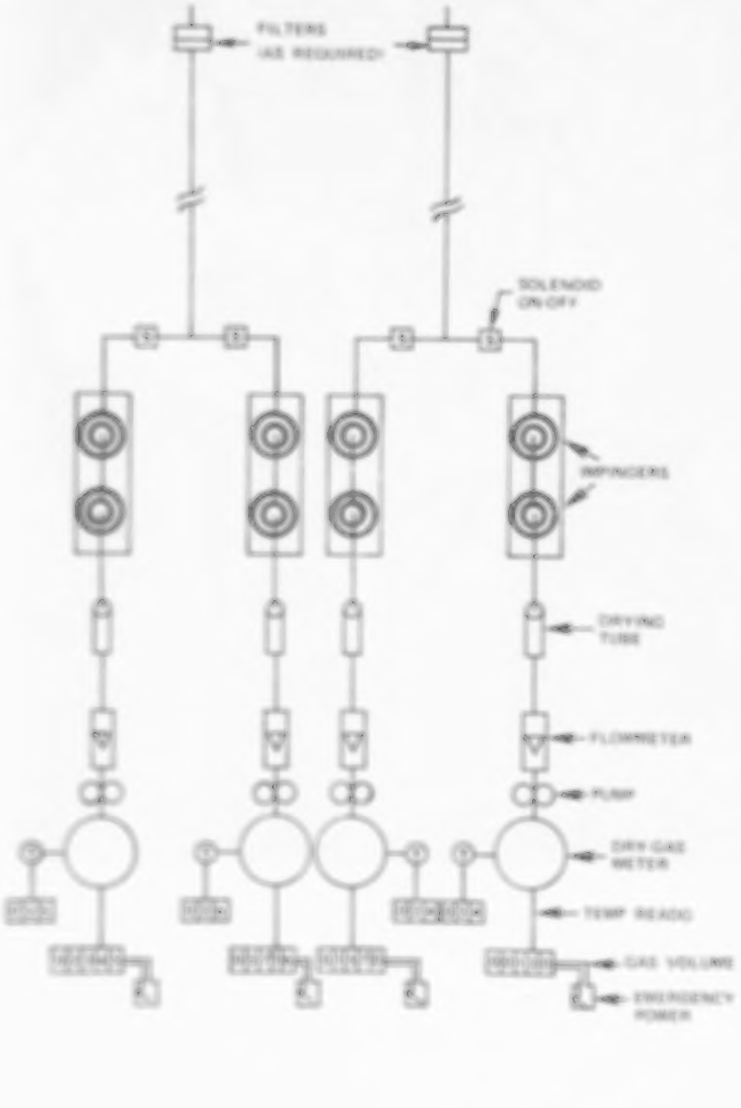


FIGURE 4 UNREGULATED EMISSIONS SAMPLING

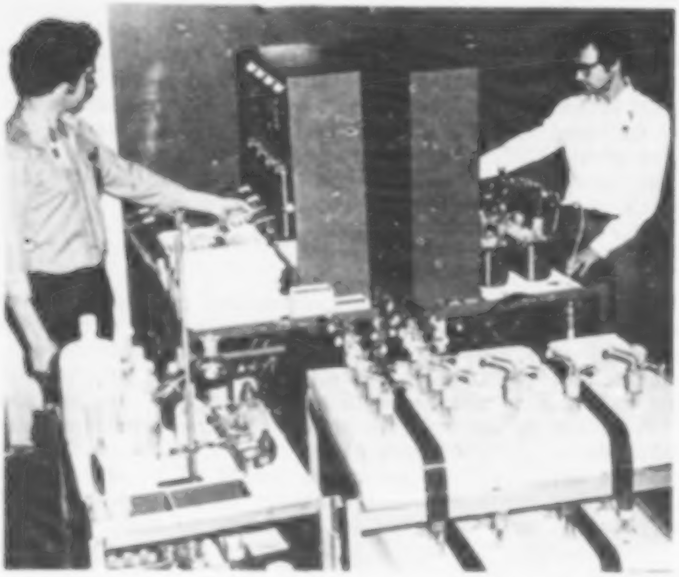


TABLE 2 CHEMICAL METHODS

UNREGULATED EMISSION	ANALYSIS METHOD
1. AMMONIA	ION CHROMATOGRAPHY
2. ALDEHYDES	HPLC
3. HYDROCARBONS	
A) TOTAL	FID
B) PART. FRAC.	GC/MS
C) PART. FRAC.	COAM
D) POST FILTER	COAM/GC
4. TOTAL CYANIDE	GC/MS
5. HYDROGEN SULFIDE	COLORIMETRIC/UV (Methylene Blue)
6. METALS	AA//X-RAY FLUOR.
7. N-NITROSAMINES	GC/MS
8. ODOR	COAM
9. PARTICULATE	GRAVIMETRIC BALANCE
10. SOLVENT EXTRACTABLE FRACTION	GRAVIMETRIC BALANCE GC/MS//COAM
11. PNA (BaP)	HPLC//GC/MS
12. SOL. SULFATES	ION CHROMATOGRAPHY
13. SULFUR DIOXIDE	ION CHROMATOGRAPHY

Carbonyl Compounds

Initially a 2,4-dinitrophenylhydrazin (DNPH) procedure utilizing gas chromatography (19) was tried. Resolution of peaks and retention repeatability were unsatisfactory. A variation of the procedure using a modified sample work-up followed by HPLC analysis using a UV detector was developed with IITRI. Some nine carbonyl compounds were studied. Additional work on this method is continuing. An HPLC trace of some standards is shown in Figure 5.

Total Cyanide

The measurement of hydrogen cyanide and cyanogen in diesel exhaust is accomplished by collecing exhaust in two glass bubblers in series maintained at ice bath temperature, each containing 25 mL 1.0 N KOH. An aliquot of the absorbing solution is treated with potassium phosphate and chloramine-T. In Reference 15 a portion of the resulting cyanogen chloride is analyzed by a gas chromatograph with an electron capture detector. We are using the GC/MS in place of the E.C.D. Our minimum detection limit is 0.1 ppm in solution. No cyanides were detected in the tests to date.

Hydrogen Sulfide

The measurement of H2S in exhaust gas is accomplished by collecting exhaust in two glass bubblers in series maintained at ice bath temperature. The absorbing solution is buffered zinc acetate solution which traps the sulfide ion as zinc sulfide. The absorbing solution is treated with N,N dimethylparaphenylene diamine sulfate and ferric ammonium compound, methylene blue. The colored solution is analyzed by spectrophotometer at 667 nm in a 1 cm pathlength cell. Our minimum detectable concentration is 0.1 ppm in solution.

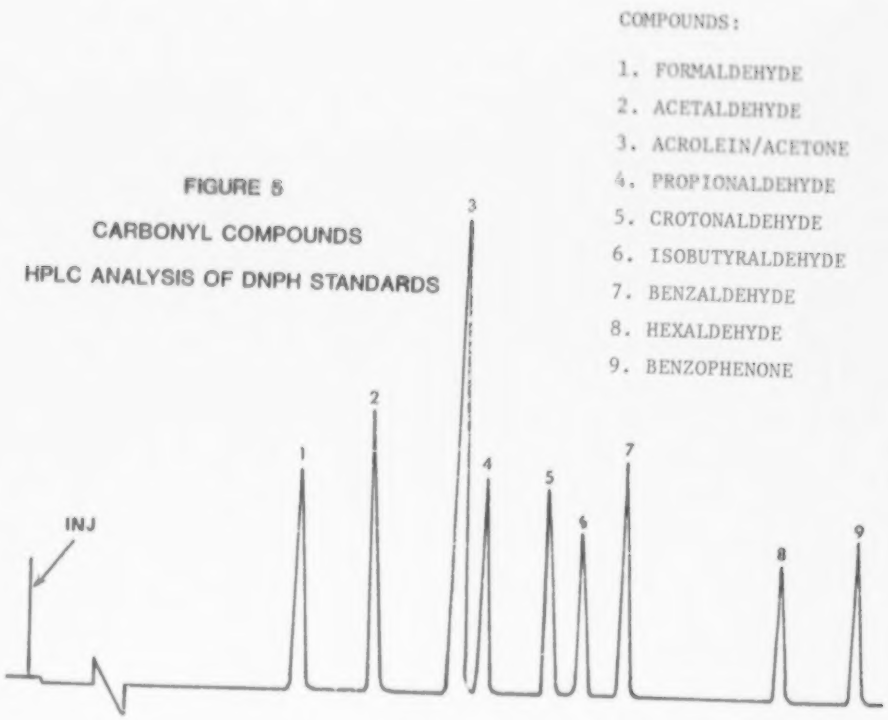
Metals

An initial survey of several samples was conducted. A NIOSH procedure (18) using atomic absorption (AA) detection methods was used. The samples were collected on 0.8 μ cellulose acetate filters, Millipore Type AA, 44 mm, digested with nitric and perchloric acids and analyzed by AA. Most metals with the exception of traces of Zn and Ca were not detected. X-ray fluorescense is currently under study to verify the results.

FIGURE 5

CARBONYL COMPOUNDS

HPLC ANALYSIS OF DNPH STANDARDS



COMPOUNDS:

1. FORMALDEHYDE
2. ACETALDEHYDE
3. ACROLEIN/ACETONE
4. PROPIONALDEHYDE
5. CROTONALDEHYDE
6. ISOBUTYRALDEHYDE
7. BENZALDEHYDE
8. HEXALDEHYDE
9. BENZOPHENONE

Nitrosamines

An initial attempt was made to detect the presence of dimethylnitrosamine (DMNA) and dibutynitrosamines (DBNA). Samples were collected at two engine conditions on filters and Tenax^R GC traps. These were eluted or thermally desorbed and examined at IITRI using SIM GC/MS. No nitrosamines were detected. Samples of DMNA and DBNA standards processed similarly showed detected limits in the ppb range. However, the smapling procedures for nitrosamines were not considered adequate and further work may be required to validate our sampling method.

Particulate

Measurement of total particulate was conducted by sampling both direct and diluted exhaust. Initial procedure development involved direct sampling of engine exhaust to expedite collection of samples.

However, all particulate data reported on the three heavy duty diesel engines were obtained using diluted exhaust sampling. Samples were obtained using 47 mm and 70 mm diameter Teflon coated glass fiber filters.

Solvent Extractable Fraction (SEF)

The diesel particulates in this study were collected on Teflon coated glass fiber filters. The amount and composition of the extractables depends on the extraction procedure and the solvent used (16,17). In this study the SEF refers to material extracted by methylene chloride. Soxhlet extraction for 6 hours at 6 cycles per hour using 20 mL of solvent was normally used. The SEF was further processed for PNA analysis and in some cases characterized by liquid chromatography (LC) or gas chromatography-mass spec (GC/MS).

Benzo-oc-Pyrene

Analysis for BaP, a suspected carcinogen, was performed on the SEF of all engine samples. The procedure was developed under contract by IITRI and utilizes HPLC with fluorescence detection. The procedure involves concentration of the SEF, fractionation on silica gel, concentration of the PNA fraction and a solvent change prior to analysis HPLC. The HPLC analysis is on a DuPont Zorbox ODS reverse-phase column with an acetonitrile-water mobile phase. Elution is isocractic and components are detected by fluorometer. The fluorescent detector is set at $\lambda ex = 280$ and has an emission cutoff filter at $\lambda em > 389$. Using this procedure fourteen PNA's were studied, Table 3. The BaP analysis was quantified and used to measure BaP in the engines.

Soluble Sulfate

Sulfuric acid aerosols and other sulfates in diesel exhaust are collected on a fluorocarbon membrane filter. The soluble sulfates are leached from the filter with water and analyzed by ion chromatography as was sulfur dioxide. The recommended procedures in Reference 15 converts the soluble sulfates to ammonium sulfate which is analyzed by a barium chloranilate procedure using high performance liquid chromatography. Our procedure is reliable and less time is required for sample work-up.

Sulfur Dioxide

The concentration of SO_2 in diesel exhaust is measured as sulfate using the ion chromatography. Exhaust samples are collected in two glass bubblers in series maintained at ice bath temperature, each containing 25 mL of 3% hydorgen peroxide. The samples are then directly analyzed on the ion chromatograph and compared to standards of known sulfate concentrations.

MEASUREMENT AND CHEMICAL CHARACTERIZATION OF SOLVENT EXTRACTABLE FRACTION (SEF)

Chemical characterization of complex mixtures such as diesel exhaust can involve identification and quantification of specific compounds such as the PNA's or it can quantify fractions or classes of compounds. In the latter case the fractionation depends on the chemical separation process employed. In this study a LC technique was used to characterize the SEF. It is a modification of an odor analysis method (COAM) used by this laboratory. Essentially the percent aromatic hydrocarbons and percent polar compounds or "oxy" compounds were determined using the system shown on Figure 6A. Typical analyses using UV fluorescence at 254 nm are shown on Figure 6B. The first peak in each pair represents the aromatic content, the second, the "oxy".

TABLE 3

RELATIVE RETENTION TIMES OF VARIOUS PNA RELATIVE TO FLUORANTHENE

PNA	Retention Ratio
Acenaphthene - (Acn)	0.70
Phenanthrene - (Ph)	0.74
Anthracene - (An)	0.79
Fluoranthene - (Fla)	1.00
Pyrene - (Py)	1.14
Benz[a]Anthracene - (BaA)	1.39
Chrysene - (Chr)	1.38
Benz[e]pyrene - (BeP)	1.96
Perylene - (Per)	1.96
Benzo[k]fluoranthene - (BkF)	1.96
Benzo[a]pyrene - (BaP)	2.23
Dibenz [a,h]anthracene - (DBahA)	2.52
Benzo[g,h,i]perylene - (BghiP)	3.25
Dibenz[a,i]pyrene - (DBaiP)	4.96

FIGURE 6a LIQUID CHROMATOGRAPH

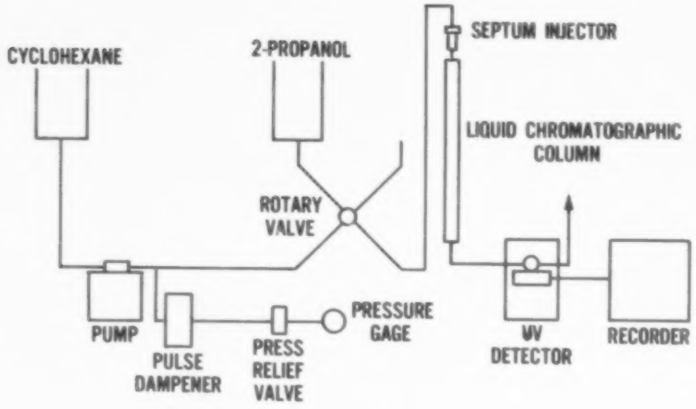
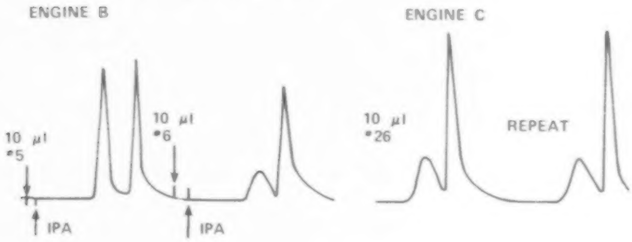


FIGURE 6b TYPICAL LC (COAM) ANALYSES

COLUMN: CORASIL II; 50cm x 2mm

SOLVENTS: HEXANE; IPA



The paraffin-naphthene fraction was determined by subtracting the total of the aromatic and oxy fractions from the SEF. The COAM can also be modified to separate a transition fraction. Similar separations can be obtained using HPLC but require considerably more effort. A comparison of the COAM and HPLC method is shown on Table 4.

In addition to the above procedure the SEF was analyzed by GC/MS and the molecular weight distribution compared to the fuel.

ENGINES AND FUEL

Engines

Three engines were used in this study and are described in Table 5. They are typical production heavy duty diesel engines. Engines A and B are similar engines. Engin B has exhaust gas recirculation (EGR) for NO_x control. Prior to an unregulated emission test the engines were run on the 13-Mode Federal Test Cycle to check the emissions and insure typical performance.

Fuel

The fuel used in the tests was a typical No. 2 diesel fuel. Since the fuel came from a central system, samples were taken prior to and at the completion of each test for comparison by GC/MS. A typical total ion chromatogram of the fuel trace is shown in Figure 7. Typical physical properties of the fuel are shown in Table 6. No evident change in the physical properties of the fuel was noticed from test to test. Some change in BaP content was noticed.

13-Mode Cycle

All engine samples were obtained at stead-state engine operating conditions. The individual 13-Mode Federal Test Cycle engine modes were analyzed and a weighted composite value calculated.

TABLE 4 SEF CHARACTERIZATION

LC VS. HPLC

		LC (COAM), %			HPLC-UV, %		
SAMPLE	METHOD*	AROM	TRANS	OXY	AROM	TRANS	OXY
HEAVY DUTY DIESEL	1	26.7	-	73.2	-	-	-
	2	26.8	15.2	58.0	-	-	-
	3	30.8	17.2	51.8	34.8	22.5	42.7

TIME REQUIRED FOR ANALYSIS, MINUTES:

METHOD	LC (COAM)	HPLC
1	3	-
2	5	90
3	7	90

*METHOD:

1 - CORASIL-II; NORMAL SOLVENT SEQUENCE FOR LC
2 - CORASIL-II; DELAYED SOLVENT SEQUENCE FOR LC
3 - PORASIL-C; DELAYED SOLVENT SEQUENCE FOR LC

TABLE 5 HEAVY DUTY DIESEL ENGINES

ENGINE	A	B	C
TYPE	DINA	DI-EGR	DITA
NO. CYLINDERS	8	8	6
BORE & STROKE, mm	114X127	114X127	137X165
RATED SPEED, RPM	2800	2800	2100
RATED POWER, Kw	157	149	284
FEDERAL TEST CYCLE DATA, G/BHP-HR:			
CO	5.92	5.37	2.20
HC	0.77	0.57	0.27
$HC+NO_2$	8.89	5.73	7.93

DI = DIRECT INJECTION
NA = NATURALLY ASPIRATED
EGR = EXHAUST GAS RECIRCULATION
T = TURBOCHARGED
A = AFTERCOOLED

FIGURE 7 FILTER EFFICIENCY CHECK

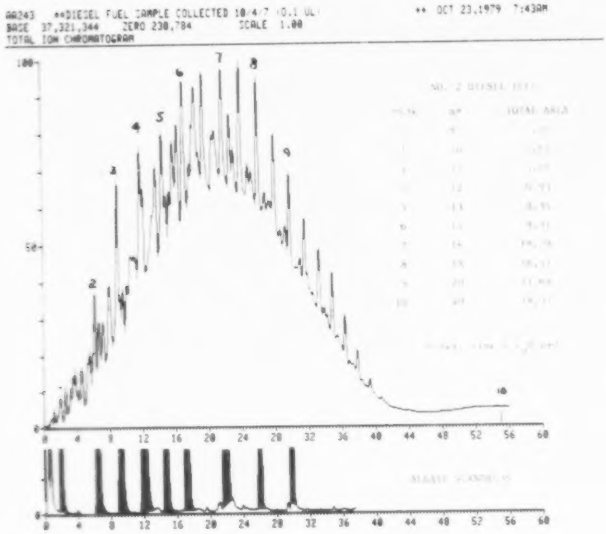


TABLE 6 TYPICAL FUEL PROPERTIES

		DISTILLATION, TEMP °C	
API GRAV	35.2	IBP	354
% SULFUR	0.27	5	394
% CARBON	85.5	10	412
% HYDROGEN	12.9	50	494
% AROMATICS	34.4	90	583
% OLEFINS	0.8	95	611
% PARAFFIN/NAPHTENES	64.8	E.Pt.	629
VIS, SSU @ 210°F	1.03	REC, %	98

EXPERIMENTAL RESULTS

Particulates

The ability to reproducibly obtain diesel particulate samples was studied. The variables studied include:

- Type of filter medium,
- Weighing precision and accuracy,
- Filter handling, drying and weighing,
- Water and fuel efefcts, and
- Filter efficiency.

In this study both T60A20 and TX40H120WW filters were used. They are vey similar Teflon coated glass fiber filters. The Pallflex TX40H120WW filters appear to be more efficient for particulates. The filters are compared on Figure 8 and Table 7. Fluoropore filters were also tried but had the highest pressure drop limiting teh size of particulate sample collected. Fluoropore filters are used for sulfate analysis sampling. Glass fiber filters reportedly (16) react with collected PNA but have been pretreated and used effectively (17).

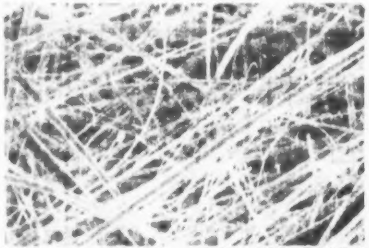
All weighings were conducted on microbalances in the same laboratory. Humidity in the lab ranged from 35 to 65% over the period of the study while temperature was maintained at 22 ± 2°C. Under these conditions there is no problem in weighing filters after storing them in a desiccator for up to two weeks before using them. Storage in a desiccator overnight was sufficient time to equilibrate filters even when spiked with moisture and raw No. 2 diesel fuel. Storage of equilibrated samples for up to three weeks did not significantly affect the results.

Serious consideration was given to normal engineering tolerances in establishing our analytical procedure. The resulting protocol used in this study is shown on Figure 9.

Particulate values obtained from the three engines are shown in Table 8. Both the composite values (G/BHP-HR) and the range of values for individual modes (G/HR) are shown. The SEF was obtained using methylene chloride. The particulate values are similar to those obtained by others studynig heavy duty diesel engines (11,20). Characterization of the SEF on two of the engines is discussed later. One item of concern is how to assess the risk associated with these levels. In some cases OSHA values or TLV recommendations may give some guidance. Another approach is to conduct

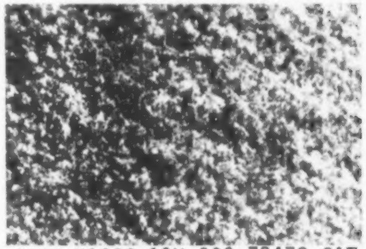
FIGURE 8 TEST FILTERS – 1000x MAGNIFICATION
(SEM PHOTOGRAPHS)

GELMAN TYPE G/A (1Mm)



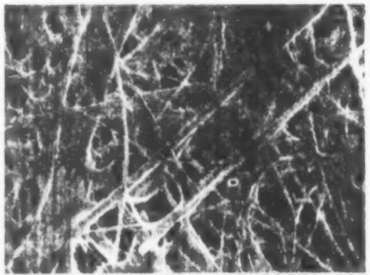
20KV x X1000 10U 002 79130 CAT

T60A20 (WHITE) PALLFLEX



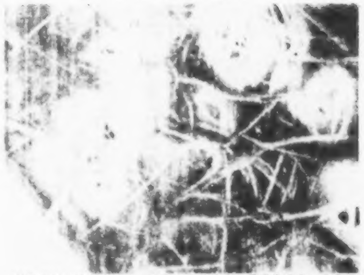
20KV X1000 10U 009 78472 CAT

PALLFLEX TX40 H120 WW



20KV X1000 10U 015 78472 CAT

FLUOROPORE



20KV X1000 10U 012 79001 CAT

TABLE 7 FILTER EFFICIENCY CHECK

TEST CONDITIONS: 2 FILTERS IN SERIES; 70 mm FILTERS; DILUTED EXHAUST.

CONDITION	HC, ppm	SMOKE, % OPACITY	% PARTIC. ON 2ND FILTER	
			T60A20	TX40H120WW
1*	322	27	6.3	0.1
2**	208	<2	9.9	3.8

* 25 L/MIN FLOW

** 55L/MIN FLOW

FIGURE 9 CHEMICAL CHARACTERIZATION BY LC (COAM)

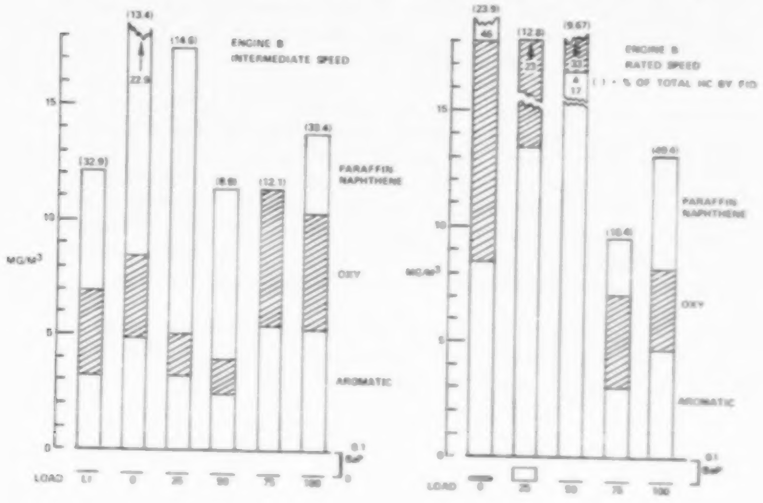


TABLE 8 PARTICULATES

ENGINE	TOTAL PARTICULATES		SOLVENT EXTRACT FRACTION		
	G/BHP-HR	RANGE, G/HR	G/BHP-HR	%	RANGE, %
A	0.77	4.6 - 200	0.19	25	6 - 92
B	1.21	2.7 - 268	0.079	6.5	2 - 89
C	0.33	1.5 - 134	0.037	11	5 - 61

evaluations using established air models and compare the results with available data. As a first pass an EPA Hiway Model (21) was used to evaluate the composite and other selected values. Using an unlikely truck population density and extreme model conditions the maximum particulate exposure standing next to a highway with bumper to bumper traffic with idling trucks was < 0.05 µg/m³. Values calculated for other species and conditions were less.

Sulfur Compounds

The SO_2 and SO_4 values are shown on Table 9. As expected the sulfur values were consistent with fuel consumption. Approximately 90% of the fuel sulfur was accounted for in the analyses. This compares to 95% reported for gasoline vehicles in Reference 15. Less than 1% of the fuel sulfur was found as soluble sulfate.

Hydrogen [illegible] would not be expected to be present at non-catalyzed, lean operating conditions of the diesels. Under our conditions, detectability is 0.01 ppm in solution or about 0.1 ppm in the undiluted exhaust. H_2S was not detected at this level but may be masked by interference from SO_2 in the exhaust. The 0.1 level is well below the recommended 10 ppm TLV level. H_2S also has a strong offensive odor detectable at about a 0.1 ppm level.

Carbonyl sulfide (COS) would be formed under the same conditions that favor H_2S formation. Based on the H_2S results, samples were not taken for COS analysis.

Ammonia

This compound would not be expected in the diesel exhaust but was detected at levels of 0.77-1.15 mg/BHP-hr. However, background levels or interferences with the method produced positive results of about the same magnitude. The maximum corrected concentration found in the exhaust, 0.4 mg/m³, whether real or not is well below allowable recommended (TLV) levels, 35 mg/m³.

PNA

The procedure has the potential for detecting a number of PNA but to date most of our work has involved BaP and to a

TABLE 9 SULFUR COMPOUNDS

ENGINE	SO_2 G/BHP-HR	SO_4 mg/BHP-HR	H_2S ppm
A	0.80	8.08	< 0.1
B	1.01	10.2	< 0.1
C	0.91	17.2	< 0.1

TABLE 10 BaP by HPLC

ENGINE	µg/BHP-HR	RANGE, mg/Kg FUEL	FUEL BaP, mg/Kg FUEL
A	1.08	0.001-0.021	4.7
B	4.36	0.001-0.100	16.6
C	0.36	<0.001-0.021	12.7

TABLE 11 EXHAUST: FUEL BaP

	µg BaP/BHP-HR	mg BaP/Kg FUEL	RATIO*
ENGINE A	1.08	4.7	0.0012
ENGINE B	4.36	16.6	0.0014

$$\text{*RATIO} = \frac{\text{BaP OUT IN EXHAUST}}{\text{BaP IN FUEL IN}}$$

lesser extent BaA. The GC/MS has been used to verify BaP and BaA values. The composite or cycle BaP values in mg/BHP-hr for the three engines are shown on Table 10. The range of BaP values in Mg/Kg fuel burned is shown for the 11-Modes of the 13-Mode cycle. The BaP values are consistent with reported levels (11,22). However, fuel BaP values appear to be considerably higher than reported values from a survey of some 20 diesel fuels (22). The engines are efficient in burning the fuel but some of the exhaust BaP could have originated in the fuel as indicated by the ratio in Table 11.

Characterization

The preceeding results are part of our effort to establish a data base for unregulated emissions. The procedures used are similar to those used by a number of laboratories and the engines tested and scheduled for testing are representative of our various engine families. In addition to this effort we have also been evaluating other ways of characterizing the emissions, especially the particulates. We have looked both at a modification of the Caterpillar LC odor analysis method (CDAM) and GC/MS to evaluate the SOF.

The results of the LC method is shown on Figures 10 and 11 for Engines B and C. The difference between the distribution of oxy and aromatic fractions is obvious. The transitional fraction referred to by EPA would be part of the "oxy" fraction. Preliminary results to break this fraction out are shown in Table 8. The BaP is a minor portion of the aromatic fraction as shown in Figures 10 and 11.

The same fractions shown in Figures 10 and 11 were characterized by GC/MS for molecular weight distribution and comparison with diesel fuel. Some typical results are shown in Figures 12 and 13 and Table 12. The results are in general agreement with those reported by F. Black of EPA-RTP (3). The fractions are essentially in the upper molecular weight range of the fuel with some higher molecular weight material either formed by polymerization or originating in the lubricant. The traces for [illegible]-load and [illegible] fuel shown on Figure 12 are typical of those containing larger fractions of higher molecular weight material. BaP on these traces would elute at about 42 minutes.

Tests were conducted at Mode 11 using a hot (190° C) filter to obtain a SOF for comparison with a SOF fraction taken at 52° C. The samples were taken from the dilution tunnel, Figure 14, and the same protocol followed. The SOF from the hot (190° C) particulate sample is less than a tenth of the

FIGURE 10 CHEMICAL CHARACTERIZATION BY LC (COAM)

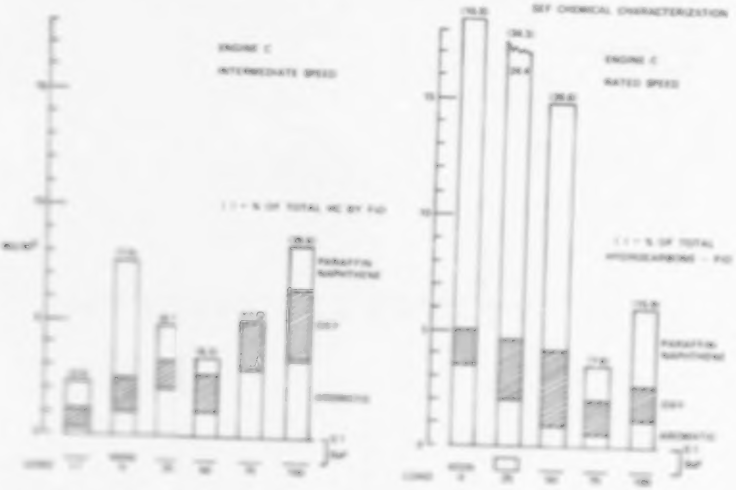


FIGURE 11

PROTOCOLFOR CHARACTERIZATION OF PARTICULATES

1. PLACE FILTER IN DESICCATOR UNTIL READY TO USE. MINIMUM – OVERNITE
2. WEIGH FILTER
3. DESICCATE UNTIL DAY OF USE
4. COLLECT PARTICULATE SAMPLE
5. DESICCATOR OVERNITE AS MINIMUM, KEEP IN DARK OR REDUCED UV LIGHTING
6. WEIGH FILTER · PARTICULATE
7. SOXHLET EXTRACTION, $MeCl_2$, 8-HOURS, 6 CYCLES/HR. IF UNABLE TO EXTRACT SAME DAY, STORE IN DARK IN FREEZER (-20°C)

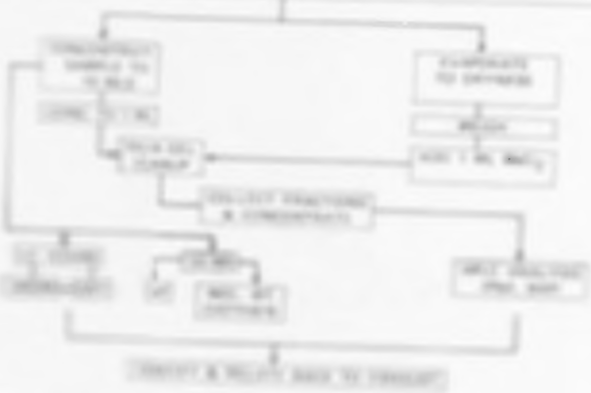


FIGURE 12 CHEMICAL CHARACTERIZATION BY GC/MS

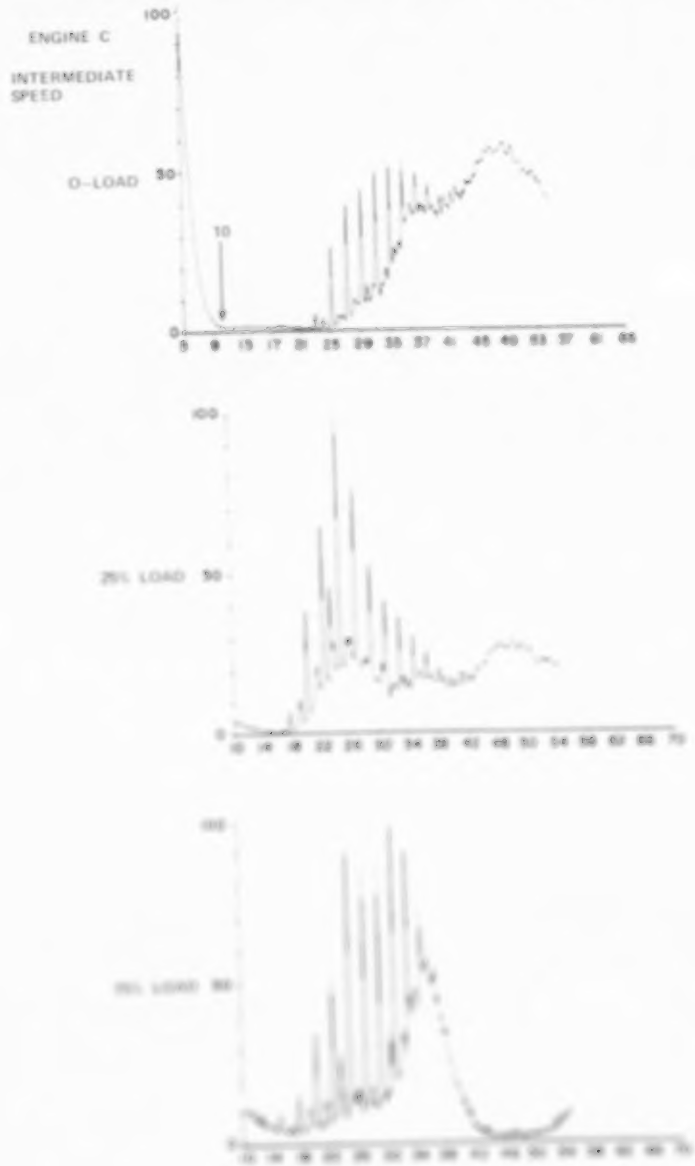


FIGURE 13

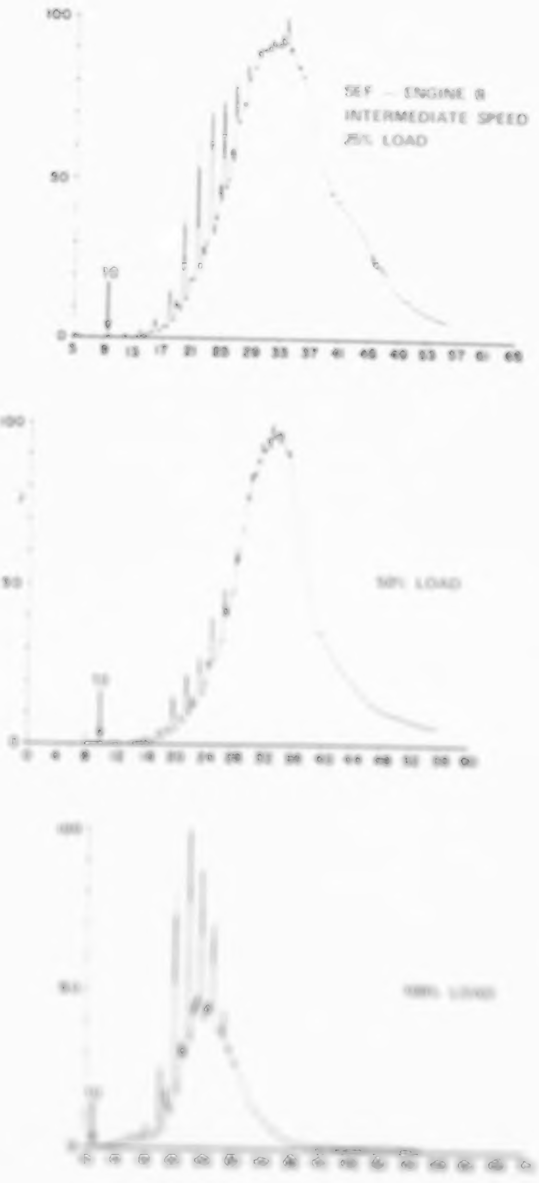

100
50
0
10
5 9 13 17 21 25 29 33 37 41 45 49 53 57 61 65
SEF – ENGINE 8
INTERMEDIATE SPEED
25% LOAD
100
50
0
10
50% LOAD
100
50
0
10
100% LOAD

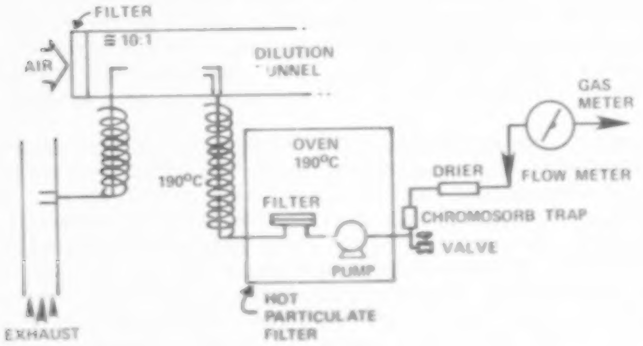
TABLE 12 GC/MS SEF FRACTIONIZATION

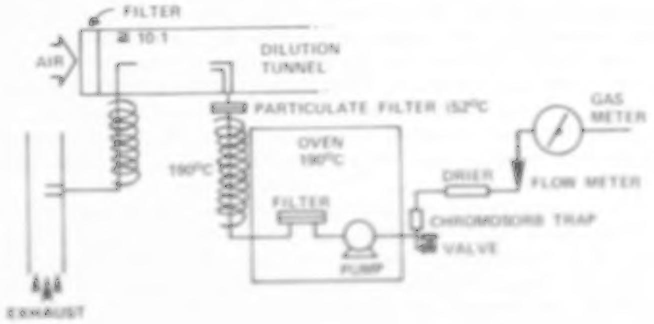
	$\Delta\% \leq CnH_2n+2$				
	C_{12}	C_{14}	C_{16}	C_{20}	C_{40}
FUEL	15.9	18.0	23.3	30.0	12.8
ENGINE B					
MODE 2	0.5	0.3	1.3	16.8	81.1
3	0	0.2	2.5	19.7	77.8
3*	0	0.2	0.6	3.4	62.5
3**	5.2	32.2	33.2	24.3	5.1
4	0.0	0.1	0.4	7.3	92.2
5	0.2	3.6	6.4	33.4	56.4
6	0.0	1.4	4.0	58.3	35.8
7	1.2	1.2	1.2	12.9	83.5
8	0.5	0.8	3.4	40.3	55.1
9	0	1.1	3.6	21.4	73.9
10	0.2	2.2	8.3	24.9	64.4
11	0.2	0.2	0.7	16.7	82.2
12	0	0.1	1.0	12.2	88.7

* [illegible] FILTER

** TRAP AFTER 52°C FILTER

FIGURE 14 HOT & COLD PARTICULATE SAMPLING





SEF from the cold (52° C) particulate sample, Table 13. The GC/MS trace, Figure 15, showed loss of the "lighter" material. This was similar to data reported in 1968 (7) at which time vaporization of hydrocarbons from the filter of direct exhaust samples was suggested. This study would tend to agree that the hot FID analysis for total hydrocarbons accounts for about 95% of all hydrocarbons in the exhaust.

Recently, Johnson et al. (23) in characterization studies of particulates indicated appreciable amounts of hydrocarbon can be adsorbed on the surface of some particulates. In addition, hydrocarbon aerosols can readily be formed on cooling. An inability to obtain a hydrocarbon material balance in the preceeding experiments and our earlier characterization studies suggested a significant unaccounted fraction, resulted in two additional experiments at Modes 3 and 11. The tests were conducted using a Chromosorb 102 trap after both hot (190° C) and cold (52° C) filters shown on Figure 16. It was found, Table 14, that in some tests more solvent extractable material is passing through the filter than is collected on it. Of even more significance the BaP levels on the Chromosorb can exceed that of the SEF. The molecular weight distribution for the fractions trapped on the Chromosorb are compared to the SEF on Figure 16. The SEF trace shown is for the material trapped on the filter upstream from the Model-11 (25% load, rated speed) chromosorb trap. The traces shown are for the corresponding Mode-3 and Mode-11 samples shown on Table 14. Attempts to validate the results and establish the variables affecting the split are in progress. The work raises some very significant questions regarding the extensive amount of risk assessment work underway using the $MeCl_2$ extractable particulate fractions. Are we testing the right material? Are some of the differences between laboratories in short term tests and animal tests due to differences in particulate collection methods?

SUMMARY AND CONCLUSIONS (FIGURE 17)

This work was an attempt to obtain baseline data on unregulated emissions for some heavy duty diesel engines. In comparison to established OSHA and ACGIH values none of the substances we have measured to date are present in the exhaust at concentration levels that could be considered dangerous at least for short term exposures.

The complexity of the exhaust and the time consuming effort required to conduct unregulated emission analyses suggest the need for prioritization with characterization of the particulate and solvent extractable fraction having high

TABLE 13 HOT & COLD PARTICULATE SAMPLES

CONDITIONS INCLUDE:

MODE II
SAMPLING AS SHOWN ON FIGURE 14

	HOT,* DILUTION TUNNEL	COLD,* DILUTION TUNNEL
TOTAL PARTICULATE, G/HR	95.3	107.6
SOLVENT EXTRACTABLE FRACTION, G/HR	0.56	9.59
%	0.59	8.9

* HOT = 190°C
COLD = 52°C

FIGURE 15 SEF-GC/MS

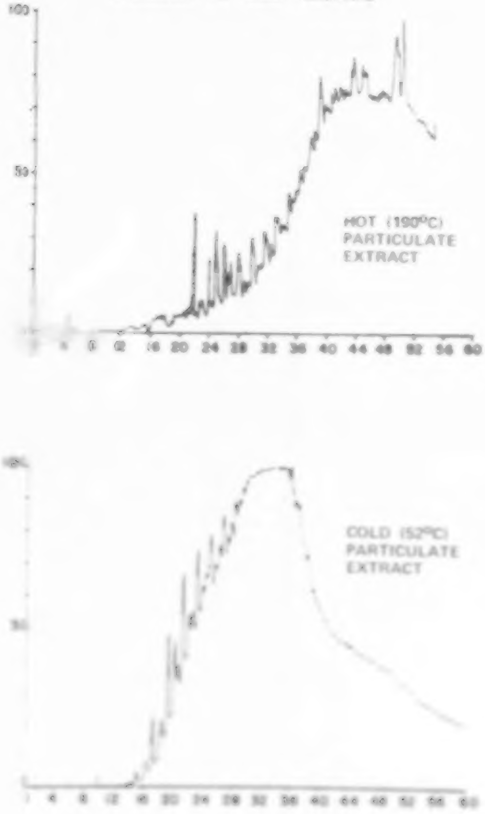


FIGURE 16 CHEMICAL CHARACTERIZATION OF TRAP PRODUCTS

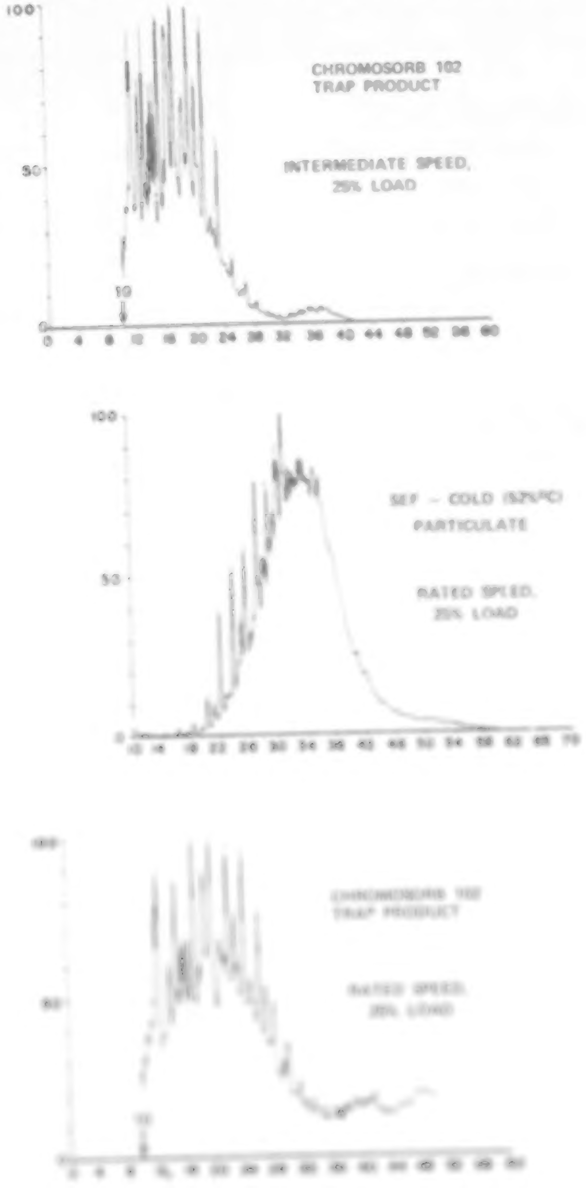


TABLE 14 CHROMOSORB TRAP PRODUCT

SAMPLING AS SHOWN ON FIGURE 14

	MODE 3	MODE 11
COLD FILTER (52°C)		
PARTICULATES, G/HR	17.9	107.6
SEF, G/HR	9.67	9.59
BaP, mg/HR	0.10	5.25
CHROMOSORB TRAP		
SOLVENT EXTRACT, G/HR	18.8	25.7
BaP, mg/HR	2.2	1.9
HOT FILTER (193°C)*		
PARTICULATES, G/HR	7.04	125.2
SEF, G/HR	0.05	0.35
BaP, mg/HR	--	1.31
CHROMOSORB TRAP		
SOLVENT EXTRACT, G/HR	25.0	15.4
BaP, mg/HR	0.41	0.41

*UNDILUTED EXHAUST

FIGURE 17

MEASUREMENT OF UNREGULATED EMISSIONS – SOME HEAVY DUTY DIESEL ENGINE RESULTS

SUMMARY AND CONCLUSIONS

- BASELINE UNREGULATED EMISSIONS DATA ON SOME HEAVY DUTY DIESEL ENGINES
- NO UNUSUAL CONCENTRATION LEVELS
- VALIDATION OF METHODS
- CHARACTERIZATION OF SEF – HIGH PRIORITY, IN PARTICULAR:
  1) ORIGIN OF CONSTITUENTS
  2) SIGNIFICANCE OF MATERIAL PASSING THROUGH FILTER
- BEST JUDGEMENT IN RELATING TO RISK ASSESSMENT:
  1) MEASUREMENT
  2) MODELS
  3) ENGINEERING TOOLS

priority. The immediate concern is to establish the significance of the material passing through the particulate filter in the gaseous phase.

Validation of the chemical methods for analysis of a number of unregulated emissions in diesel exhaust is required. The ability to measure and characterize diesel emissions remains a challenge but must be vigorously pursued if a fair assessment of the risk, problem comopunds, and benefits of control technology is to be made.

ACKNOWLEDGEMENTS

R. V. Bower, Caterpillar Research and M. Poff, Technical Facilities were instrumental in setting up our facilities. K. Claar and R. D. McDowell's cooperation on obtaining and operating the engine test and evaluation facilities for this study and the technical assistance of R. V. Bower, L. A. Schoepke, and V. J. Huisenga in the chemical areas are appreciated.

Dr. W. J. Eisenberg, IITRI, conducted method development work on PNA and aldehydes under contracts for Caterpillar. His cooperation on these and other tests was very helpful in developing our in-house capability.

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POLYNUCLEAR AROMATIC HYDROCARBONS IN DIESEL EMISSION PARTICULATES

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ABSTRACT

A preliminary characterization of polynuclear aromatic hydrocarbons (PAH) in dichloromethane extracts of diesel exhaust particulates is described. Acid-base liquid-liquid partitioning followed by adsorption chromatography was used to isolate the PAH fraction. Compounds were identified primarily by the mass spectra of high-resolution gas chromatographic effluents. Three-four-ring PAHs and their alkyl-substituted homologs were the predominant constituents. In addition, ultraviolet spectra of four high performance liquid chromatography-separated PAHs were superimposable with those of reference compounds leading to their unambiguous identification. These results show the advantage of using several complementary techniques for characterization rather than gas chromatography-mass spectrometry alone.

INTRODUCTION

Polynuclear aromatic hydrocarbons are products of various combustion processes. Most of the carcinogenic PAHs in the ambient air are associated with the particulate matter(1). The expected increase in the use of diesel powered vehicles will greatly increase the automotive contribution of particulate-adsorbed PAHs and other organics in air because these vehicles emit 30-50 times more particulates than a comparable gasoline-powered vehicle (2). Consequently it is important to characterize thoroughly the particulate-adsorbed PAHs in diesel exhausts.

Several workers have identified PAHs in diesel exhaust particulate extracts (3,4), these identifications were based primarily on data from analysis by gas chromatography-mass spectrometry (GC-MS) or thin layer chromatography and high performance liquid chromatography. Because several isomeric PAHs are difficult or impossible to separate by GC, a firm identification is difficult to achieve by GC-MS alone.

In this communication we describe preliminary results of a comprehensive characterization study of PAHs in diesel emission particulates. Most of the work was performed on a sample from a Volkswagen (VW) Rabbit driven in a highway fuel efficiency test (HFET) mode. Preliminary results from a GC-MS examination of a sample from a Mercedes 300-D exhaust (federal test procedure {FTP} driving cycle) are also included.

EXPERIMENTAL

Collection of Particulates and Preparation of the Organic Extract

Diesel-exhaust particulates were collected by a modification of the standard dilution-tunnel technique. The Pallflex T60A20 Teflon-coated glass fiber filters (20" x 20") containing the particulates were Soxhlet extracted with dichloromethane for 24 h. The extract was filtered through a 0.2-μm Fluoropore filter under vacuum, and the solvent was removed under vacuum with gentle heating. A composite of several filter extracts was prepared for chemical characterization. Details of the sampling procedure and extraction are given elsewhere in this Proceeding (5).

Isolation of the PAH Fraction

The crude extract is a highly complex mixture. To isolate the PAHs from interfering non-PAH compounds, the neutral fraction was first separated by acid-base partitioning. The PAH fraction was then isolated from the neutral fraction by silica-gel adsorption chromatography (6). Fluorene and coronene were chromatographed under similar conditions to determine the elution volume of the PAH fraction.

Solvents and Chemicals

Solvents for high performance liquid chromatography (HPLC) and other uses were glass distilled, ultraviolet grade (Burdick and Jackson Laboratories, Inc., Muskegon, MI). PAH reference compounds (Aldrich Chemical Co., Eastman Kodak, and K & K Laboratories) were checked for purity and, if necessary, were purified further by column chromatography on silica gel and recrystallization from an appropriate solvent.

Glass Capillary Column for High Resolution Gas Chromatography

A glass capillary column (45 m x 0.35 mm) was drawn from Pyrex glass tubing using a Shimadzu capillary drawing machine (Shimadzu Scientific Instruments, Inc., Columbia, MD). The column was filled with hydrogen chloride gas, sealed, heated for 4 h at 350°C, and cooled to room temperature. The hydrogen chloride was blown out with dry nitrogen, and a solution of 5% 1,1,1,3,3,3-hexamethyl disilazane in toluene (5 ml) was passed through the column. The column was washed with dry toluene, dried by passing dry nitrogen, and then dynamically coated with a solution of SE54 gum in isooctane under a constant pressure of nitrogen.

GC and GC-MS Analysis

For GC analysis the PAH fraction was dissolved in a small volume of chloroform, and aliquots of 1-2 μl were injected without stream splitting into a Hewlett-Packard 5840A gas chromatograph with microprocessor controls. The injector temperature was held at 230°; the pressure at the injector head was 6 Psi. Careful optimization of the injector head pressure was critical for optimal resolution. The oven temperature was started at 110°C, and 2 min after injection a multistep temperature program was initiated, reaching a final temperature of 290°C. Nitrogen was the carrier and make-up gas. The analytical conditions were carefully optimized for best resolution, minimum analysis time, and peak sharpness.

For GC-MS analysis the same column was directly connected to the source of a Finnigan 4000 mass spectrometer equipped with an INCOS 2300 data system and operated at 70 eV. Helium was the carrier gas.

High Performance Liquid Chromatography and Ultraviolet Spectroscopy

The HPLC method for analysis of PAHs was developed during our previous work on airborne PAHs (Choudhury and Bush, manuscript in preparation). A Zorbax ODS column (4.6 mm x 25 cm; DuPont Instruments, Wilmington, DE) was used with a solvent gradient system. Two solvent systems were used: methanol-water (20-80) and methanol. Elution was started with 84% methanol-water and continued for 20 min. A gradient to 100% MeOH over 20 min was then initiated. Ultraviolet spectra of the HPLC eluates were obtained by interfacing a millisecond-scan vidicon ultraviolet spectrophotometer to the HPLC instrument. This instrument, capable of providing an instantaneous and complete ultraviolet spectrum of every HPLC eluate in a continuous mode, will be described elsewhere (Smith, Aldous, Choudhury and Bush, manuscript in preparation).

RESULTS AND DISCUSSION

Trace analysis of PAHs in environmental samples is a difficult task. Blumer and Giger observed in 1974 that even when large samples are available, as in petroleum analysis, "a complete analytical resolution of the PAH fraction exceeds the capability of any existing combination of analytical techniques" (7). Computerized GC-MS is the most powerful technique normally applied to identification of PAHs and other trace organics in a complex environmental sample.

The two-step clean-up procedure used in this work was highly effective for isolation of the PAH fraction. No interfering compounds of other type were detected in this fraction by mass spectrometry. Compounds were identified primarily by high resolution GC retention times and mass spectra of the GC-separated components. Additional evidence for the identity of certain compounds was obtained from ultraviolet spectra of the HPLC eluates and HPLC retention times. Low resolution packed columns were not used, as the superior performance of high resolution capillary columns is well established. Glass capillary columns coated with several silicone-based stationary phases (SP2100, SE30, SE52, SE54) were examined for their suitability for analysis of PAHs in diesel emission particulate samples. In our hands the SE54-coated column gave the best resolution and peak sharpness, and the analysis time was quite short.

The GC profile of the PAH fraction from a VW Rabbit exhaust sample is shown in Figure 1. Comparison of the peak retention times with those of representative parent PAHs indicated that the major components were low molecular weight PAHs. The minor components could not be compared with a high degree of confidence. Four parent PAHs were identified: phenanthrene (peak 1), anthracene (peak 2), fluoranthene (peak 11), and pyrene (peak 12). The retention time of peak 7 corresponded with 2-phenylnapthalene. For more confirmed characterization of these and other constituents the mass spectrum of each component was determined using the same capillary GC column.

Some loss of chromatographic resolution was observed in the GC-MS work, presumably because the injector of the Finnigan GC-MS is drastically different from that of the Hewlett Packard gas chromatograph. We have found it virtually impossible to maintain maximum chromatographic resolution in GC-MS work. Chromatographic and mass spectral scan parameters were optimized separately for the best possible resolution.

The total ion chromatogram of the PAH fraction is shown in Figure 2. M/e values of the major components of all observable peaks were determined, and the parent ions were noted. Mass chromatograms of the parent ion and key fragment ions were then generated, and clean spectra were obtained by computer-assisted background subtraction. These spectra and the retention times were compared with those of reference com-

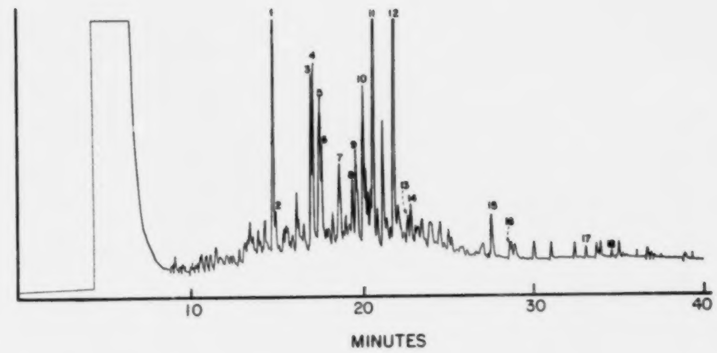

Figure 1. Gas chromatogram of PAH fraction of diesel particulate extract (VW Rabbit). GC condition: 45-m X 0.35-mm id glass capillary column coated with SE54. Flame ionization detector. Temp: 110° for 2 min, programmed to 170° at 10°/min., to 212° at 3°/min., to 278° at 7°/min., to 290° at 8°/min.

PEAK IDENTITIES

1.	Phenanthrene
2.	Anthracene
3-6.	Methylanthracene/phenanthrene
7.	2-Phenylnaphthalene
8-10.	Dimethylanthracene/phenanthrene
11.	Fluoranthene
12.	Pyrene
13-14.	Trimethylanthracene/phenanthrene
15.	Benzo(ghi)fluoranthene
16.	Chrysene Benzo(a)anthracene Triphenylene
17.	Benzofluoranthene
18.	Benzo(e)pyrene/benzo(a)pyrene

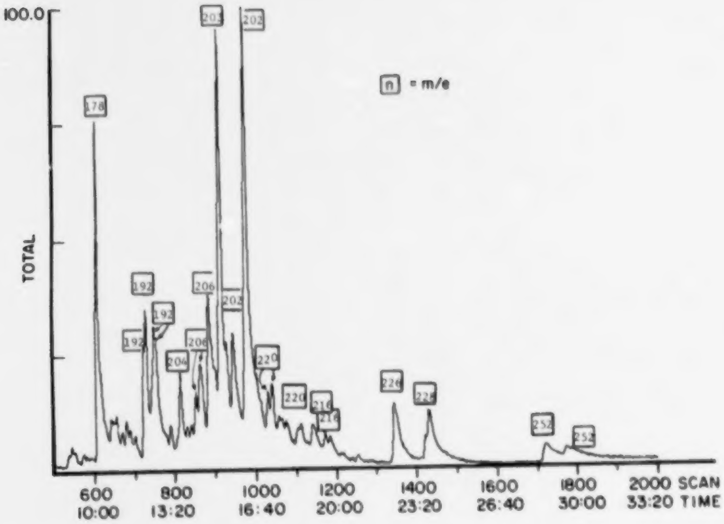

Figure 2. Total ion chromatogram of PAH fraction of diesel particulate extract (VW Rabbit).

pounds, when available. In other cases the background subtracted spectra were compared with the best-fitting spectra from the computer-stored mass spectral library. In addition to the compounds whose masses are shown in Figure 2, many minor components were characterized. A total of 45 spectra were extracted, and tentative structures were assigned.

Four compounds with parent ions of m/e 192 (also the base peaks) were present. Key fragment ions were observed at masses corresponding to $M^{\dot{+}}$-1, $M^{\dot{+}}$-27, and $M_{\cdot}^{2+}$. These spectra were in excellent agreement with those of methylphenanthrene/-anthracene. The peak with the parent ion m/e 204 was similarly identified as 2-phenylnapthalene. Three components having parent ion of m/e 206 had spectra in agreement with those of dimethylphenanthrene/-anthracene and/or ethylphenanthrene/-anthracene. At least four compounds with a parent ion of m/e 220 were detected; their mass spectra agreed with those of trimethylphenanthrene/-anthracene or methylethylphenanthrene/-anthracene. The m/e 228-parent ion peak was similarly identified as chrysene/benzo(a)anthracene/triphenylene. Gas chromatographic separation of these three components is incomplete. The mass spectrum of the peak with a parent ion of m/e 226 corresponded to that of benzo(ghi)fluoranthene, and the GC retention index of this compound (391) was in excellent agreement with that reported (389.6) by Lee et al. (8).

At least two components having parent ion of m/e 252 were detected. The first (scan 1725) was characterized as benzo(b)fluoranthene/benzo(j)fluoranthene/benzo(k)fluoranthene on the basis of mass spectrum and retention time. The retention time and mass spectrum of the second (scan 1775) corresponded to that of benzo(e)pyrene/benzo(a)pyrene. Although the GC column used can separate the two, no separate peaks were observed, presumably because of their low concentrations and loss of resolution of the gc column in GC-MS. Compounds with a higher ring system could not be detected in this sample.

Some additional minor components characterized tentatively by mass spectral data were isomeric methyldibenzothiophenes/-dibenzodioxins, isomeric dimethyldibenzothiophenes/-dibenzodioxins, benzo(a)fluorene, benzo(b)fluorene, and isomeric methylpyrenes/-fluoranthenes.

Since mass spectrometry cannot differentiate between isomeric PAHs, additional independent evidence for identification of individual isomers is desirable. This is particularly important because the carcinogenic properties of PAHs are depentant on the isomeric structure. PAHs absorb strongly in the ultraviolet region, and we took advantage of this distinctive property to achieve their unambiguous characterization. HPLC retention times could also be used for identification, as HPLC gives excellent separation of PAHs. Our ultraviolet spectra were obtained by interfacing a millisecond-scan ultraviolet spectrophotometer to the HPLC instrument. We have also used this instrumentation to obtain unambiguous identification of several PAHs in airborne particulates (Choudhury and Bush, manuscript in preparation).

The HPLC profile of the PAH fraction of the VW Rabbit sample is shown in Figure 3. The retention times of components 1, 2, 3, and 4 corresponded with those of phenanthrene, anthracene, fluoranthene, and pyrene respectively. The ultraviolet spectra of these components were superimposable on those of authentic standards of phenanthrene, anthracene, fluoranthene and pyrene respectively (Figure 4), establishing firmly their presence in the sample. Since PAHs with higher ring systems were present only in low concentrations, no effort was made to obtain their ultraviolet spectra. Work is under way to preconcentrate the higher molecular weight PAHs for firm characterization.

We have also examined the PAH fraction of the particulate extract from a Mercedes 300-D. Its GC-MS profile was very similar to that obtained from the VW Rabbit sample. Its total ion chromatogram (Figure 5) showed components characterized by GC-MS as phenanthrene, anthracene, methylphenanthrene/-anthracene, dimethylphenanthrene/-anthracene, benzo-(ghi)fluoranthene, chrysene/benzo(a)anthracene/triphenylene, benzo(b)fluoranthene/benzo(k)fluoranthene,benzo(e)pyrene benzo(a)pyrene. In this sample fluoranthene appeared as a shoulder of one of the dimethylphenanthrene/-anthracene peaks. The HPLC-ultraviolet data are not yet available.

In contrast to the diesel exhaust results, we have found that airborne particulates collected from several areas of New York State using hi-vol samplers contain qualitatively similar amounts of most parent PAHs containing 3-7 rings (Choudhury and Bush, manuscript in preparation). Very few alkyl-substituted PAHs were detected in our samples. The sample collection methods were different, however, and therefore while it is an important observation, a strict comparison between airborne PAHs and those in diesel particulates is inappropriate.

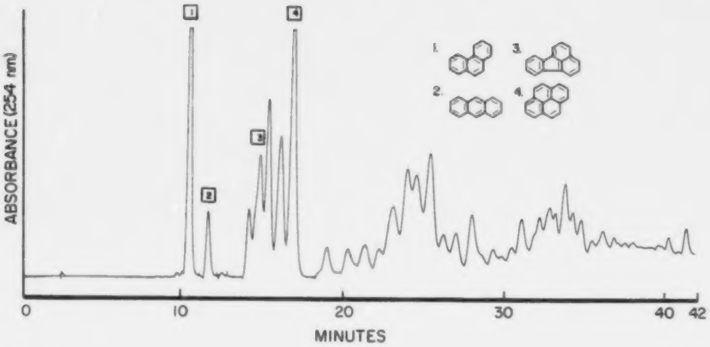

Figure 3. HPLC profile of PAH fraction of diesel particulate extract (VW Rabbit). HPLC condition: 4.6-mm x 25-cm Zorbax ODS column. MeOH/H_2O (84/16). 1.6 ml/min. Linear gradient to 100% MeOH (20min.) started after 20 min.

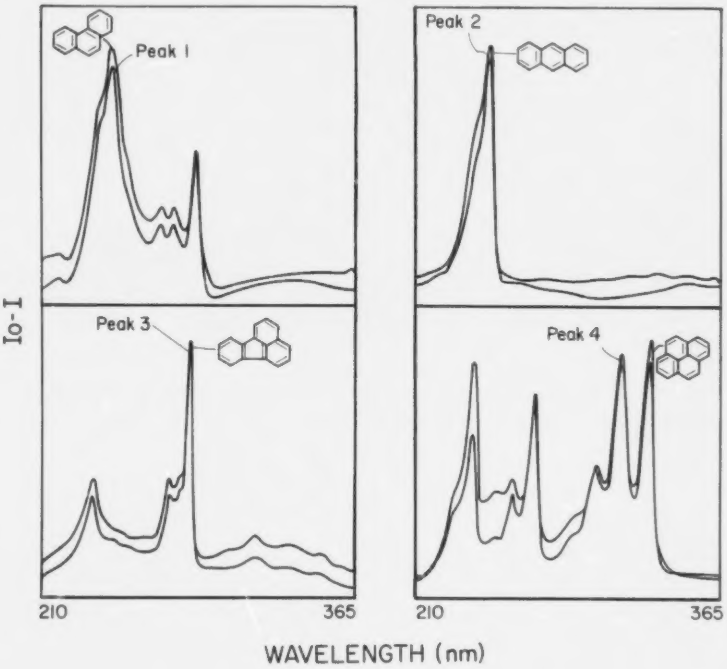

Figure 4. Ultraviolet spectra of HPLC eluates of PAH fraction of diesel particulate extract (VW Rabbit).

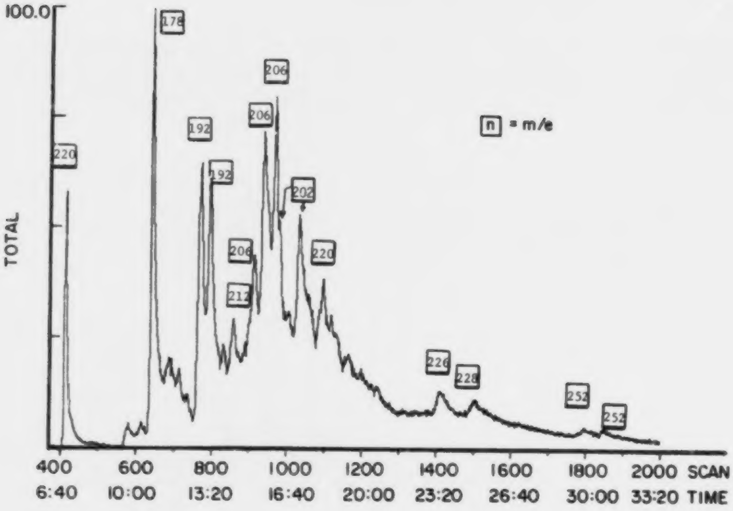

Figure 5. Total ion chromatogram of PAH fraction of diesel particulate extract (Mercedes 300-D).

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General Discussion

A. KOLBER: How were the airborne particles collected?

D. CHOUDHURY: The typical high volume samples were extracted with benzene. At that time we were using benzene. Most of the samples were collected about 1975 to 1977.

A. KOLBER: Could you describe the sampling technique further.

D. CHOUDHURY: This was a typical high volume sample using glass fiber filters.

J. HORWITZ: I didn't hear whether these were wall-coated open-tubular capillaries; what kind of capillaries were employed here?

D. CHOUDHURY: These were wall-coated capillaries, by cell fractioning.

J. HORWITZ: Were the molecular ions determined with the CI or the EI source?

D. CHOUDHURY: They were EI. We have also done CI.

D. HOFFMANN: You are comparing engine emission pollutants collected one way and urban pollutants collected another. How do you know that you are not collecting any hydrocarbons, or hardly any, and therefore no methylene. Isn't this comparison a little weak? Must you not use the same collection system in order to say that this indicates some diesel pollution. Is it not correct that you can do it only when you have the same data collection system.

D. CHOUDHURY: The question is not a fixed comparison between what is and what isn't there. It is an observation and you take the data from different sources and they are all different. Now what one sees in the air is a combination of pollutants that come from all sorts of combustion sources, so you cannot really isolate their source. Basically the idea is that exhaust contains this type of agent, whereas, typically the air sample contains that kind of agent. Gasoline has one type while other combustion samples would have another type of PH profile. This is basically an observation, and it is not really strict in saying that this has that and why do we have that.

EMISSIONS OF INORGANIC COMPOUNDS
FROM HEAVY DUTY DIESEL TRUCKS ON THE ROAD

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ABSTRACT

Experiments have been conducted to measure diesel trucks' inorganic emissions at the Nihonzaka two-lane northbound tunnel of 2 km long located 170 km west of Tokyo. Average traffic of heavy duty diesel trucks was 600 per hour during the two-day period of measurements. Truck speed was 80 km per hour. Sulfur content of fuel oil was 0.4-0/0. Measurement procedures are almost similar to the Allegheny Tunnel study by Pierson. Preliminary study done in 1972, present study was started in 1978 and will continue to 1981.

Average emissions rates of heavy duty diesel truck were found to be:

(1) Nitrogen oxides = 7-9 grams/km
(2) Sulfur dioxide = 1.5 grams/km
(3) Sulphate = 50 milligrams/km
(4) Nitrate = 3 milligrams/km
(5) Total particulates = 0.8-1.0 grams/km.

The overall sulfur dioxide conversion to sulphate of emissions was 3-0/0.

The measurements at the ambient are on the way. Sulfuric acid particulates of 2-30 microns spheres were observed by microscope on the thymol blue dye coated films exposed in the ambient 100 meters distant from the tunnel portal.

The author thanks Dr. Pierson, Dr. Witz, Dr. Perhac and EPA-RTP Officers for their assistance through valuable discussions.

INTERACTIONS BETWEEN DIESEL EMISSIONS AND GASEOUS CO-POLLUTANTS IN PHOTOCHEMICAL AIR POLLUTION: SOME HEALTH IMPLICATIONS

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ABSTRACT

A complete assessment of the health effects of diesel emissions must take into account the possible chemical and biological transformations of particulate organic matter due to reactions with the many gaseous co-pollutants which have now been unambiguously demonstrated to be present in atmospheres burdened by photochemical air pollution. These co-pollutants include the "trace" species, nitric (HNO_3) and nitrous (HONO) acids, the nitrate radical (NO_3), formaldehyde (H_2CO) and formic acid (HCOOH), as well as the criteria pollutants, ozone (O_3) and nitrogen dioxide (NO_2). Techniques for establishing the atmospheric concentrations of the trace pollutants (and their spatial and temporal variations) are briefly described, and we present results of investigations into the reactions of polycyclic aromatic hydrocarbons (PAH) coated on filters and exposed to ambient concentrations of O_3 and NO_2. Environmental health implications of these results are discussed and include the potential for sampling "artifacts" and their possible effects on the correlation (or lack thereof) between ambient PAH levels and urban lung cancer rates, as well as the problems associated with understanding the appropriate POM "dose" to be employed in animal testing and assessments of impacts on human health.

INTRODUCTION

Two major pollutants of concern in diesel exhaust emissions are gaseous oxides of nitrogen (NO_x) and particulate organic matter (POM). Nitrogen dioxide, a key constituent of NO_x, is a "criteria" pollutant per se, and also represents, through its photolysis, the only known anthropogenic source of ozone in photochemical smog. Diesel POM is emitted in the submicron, respiratory size range and like most combustion-generated particulates, contains a number of promutagenic and carcinogenic polycyclic aromatic hydrocarbons (PAH) as well as other unidentified mutagens that exhibit strong direct activity in the Ames Salmonella/mammallian microsome assay.

A comprehensive assessment of the biological effects of diesel POM emissions must consider not only their composition as primary pollutants but also the nature of the ultimate products formed (during dispersion and transport) by their physical and chemical transformations in the atmosphere.

In regions experiencing photochemical air pollution, many nitrogenous and oxygenated compounds are formed as secondary pollutants by complex photochemical and thermal reactions. Some of these compounds are known to exhibit toxic, mutagenic and/or carcinogenic effects in experimental animals. Others, as yet untested, may also present health hazards. Some of these species may be capable of reactions with components of diesel POM to form potentially hazardous products.

We believe that a reliable prediction of the future health impact of greatly increased diesel emissions will require an understanding of:

- Current ambient levels of noncriteria pollutants such as nitric acid, formaldehyde, nitrous acid, etc., in airsheds suffering from moderate to heavy photochemical smog--into which additional NO_x and POM from diesels will be introduced, and

- The chemical, physical and biological results of interaction of diesel POM with these co-pollutants, as well as with the criteria pollutants O_3, NO_2, SO_2 and CO, and, in certain instances, sulfuric acid.

These data are required in order to estimate reliably the total "dose" of pollutants to which individuals might be exposed in various types of air pollution episodes involving significant levels of diesel emissions. Obtaining such information--the task of atmospheric scientists--is particularly difficult in the case of diesel NO_x and POM emitted into photochemical smog. Not only are homogeneous gas phase processes involved but heterogeneous reactions may also be important. These include the well-known formation of secondary nitrate aerosols and a wide range of possible reactions on the surfaces of primary organic particulates.

Chemical and physical transformations may occur in the presence of sunlight, oxygen, water and a spectrum of co-pollutants during transport of diesel POM and NO_x in the atmosphere over periods of hours, days or even weeks. Present mixtures of gaseous and particulate pollutants in urban airsheds are already exceedingly complex, but they may be expected to become even more so in the future as our nation utilizes such alternatives as diesel engines, synfuel technologies, and coal-fired power plants to meet the economic challenges of the energy crisis. Clearly, the identification and determination of toxic, mutagenic and/or carcinogenic gaseous and particulate species in the polluted atmospheres of the 1980s will require a high level of effort employing sophisticated instrumentation and analytical procedures (chemical as well as microbiological). These data are required to avoid two potentially costly misjudgments; on the one hand, unnecessary economic burdens if overly stringent controls are mandated, and on the other, threats to public health if possible adverse biological impacts are not recognized and resolved in a timely, economically and environmentally sound manner.

An additional level of complexity associated with this problem is the question of possible "artifact" effects occurring during the collection, extraction and analysis of POM. As illustrated in Figure 1, if artifacts are important in these procedures, the actual "dose" of the pollutants inhaled by man may be significantly different qualitatively and quantitatively from the dose assumed to be administered to experimental animals calculated on the basis of chemical or microbiological analyses of, for example, hi-vol samples collected on glass fiber filters. This point will be considered later.

In order to best confront the challenging problems presented by these issues, a joint fundamental-applied research approach involving both simulated and real atmospheres was developed at the University of California Statewide Air Pollution Research Center (SAPRC). Certain of our programs have concentrated on the characterization of the pollutant "dose" typical of atmospheres containing photochemical oxidants. More recently, our research has been extended to include studies of POM emitted from diesel engines and other major combustion sources.

Towards these ends, we have developed and employed state-of-the-art smog chamber and spectroscopic systems to identify and measure for the first time several gaseous pollutants that have been reported or postulated to exist in photochemical smog. In the following sections we will briefly

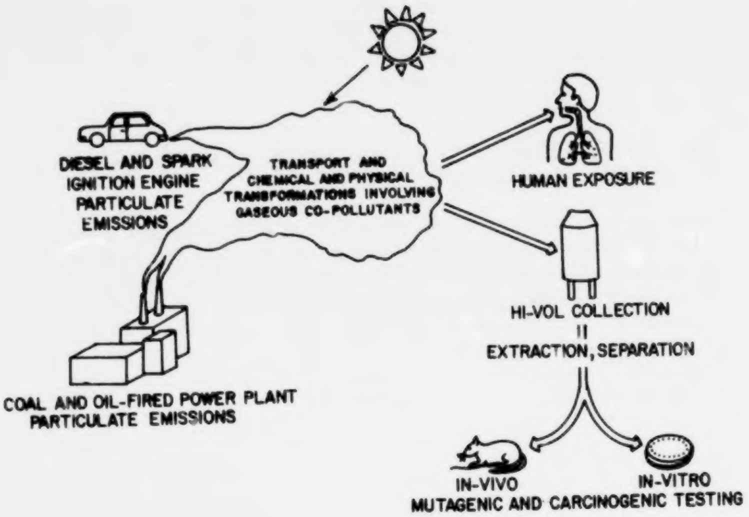

Figure 1. Chemical and physical transformations of PAH on ambient particulate matter during transport through photochemical air pollution and during collection on hi-vol filters may result in significantly different "doses" to man and animals than the POM emitted originally from primary sources.

illustrate the use of such instruments, and also consider the chemical and biological effects of interactions of a representative PAH, benzo(a)pyrene (BaP), with the "criteria" co-pollutants, NO_2 and O_3. References to original literature can be found in recent publications (1-8) from this and other laboratories.

GASEOUS CO-POLLUTANTS WITH DIESEL POM IN PHOTOCHEMICAL SMOG

The use of smog chambers fitted with long path White-cell type optics interfaced to prism and dispersive infrared spectrophotometers originated more than two decades ago. Thus, in the classic studies of Hanst, Stephens, Schuck, Doyle and their co-workers in the 1950s and early 1960s (8), a variety of compounds formed in $HC-NO_x$-air-UV systems were identified and time-concentration profiles established.

In the past decade, two major advances have been achieved in this field. One is the design of more sophisticated smog chambers and solar simulators; the other is the use of

Fourier-transform (8) infrared spectrometers. Identification and measurement of a number of important labile pollutants in simulated and ambient systems is briefly discussed in the following sections.

Smog Chamber Studies: Identification of Labile Products in Propene-NO_x-Air Systems

Irradiated mixtures of propene, NO and NO_2 in air constitute a useful and well studied model system for the photooxidation of ambient olefinic compounds (9). We have thoroughly investigated this system using our 5800-liter evacuable environmental chamber. The temperature of the chamber is controllable to $\pm$ 1°C in the temperature range from -20° to +100°C, and it utilizes a 25 kw solar simulator as a light source (Figure 2).

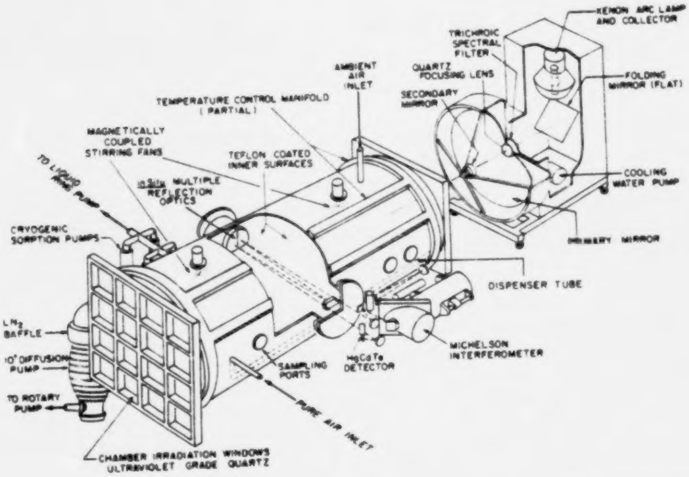

Figure 2. SAPRC evacuable smog chamber and solar simulator facility.

An Eocom interferometer, interfaced to an 85 m pathlength multiple-reflection cell in the evacuable chamber is used to obtain infrared spectra. The short scan times, high resolution, and large wave number range per scan afforded by an interferometer makes long-path FT-IR spectroscopy an ideal tool for identifying and obtaining time-concentration profiles of labile gaseous nitrogenous and oxygenated species formed in such experiments.

A high-resolution (0.125 cm^{-1}) spectrum from a run in which a mixture of propene, NO and NO_2 (at concentrations in the ppm range) in air at 9.4°C (48°F) was irradiated is shown in Figure 3. This spectrum permits identification and quantification of such species as N_2O_5, HNO_3, pernitric acid (HO_2NO_2), methyl nitrate (CH_3ONO_2) and PAN ($CH_3COO_2NO_2$). Additionally, and of importance when considering possible health effects of ambient photochemical oxidant, formic acid and formaldehyde were also observed as products.

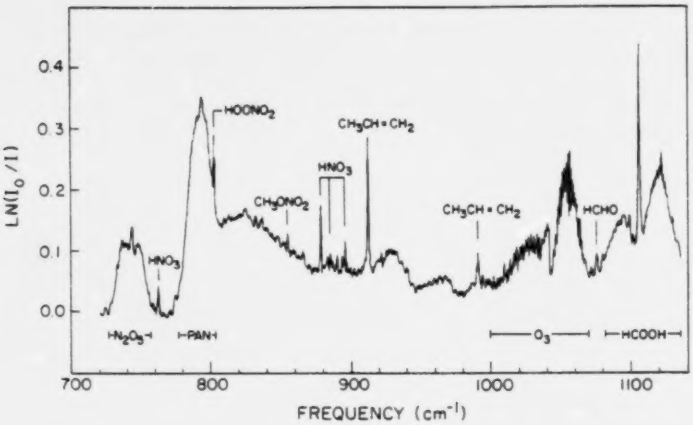

Figure 3. Nitrogenous and oxygenated compounds observed by FT-IR spectroscopy during irradiation of propene-NO_x system at 48°F in SAPRC 5800-liter evacuable chamber.

The propene system has much in common with the many other more complex systems we have studied in the evacuable chamber facility, and spectroscopic identification of the above compounds in such simulated atmospheric systems suggests their presence in ambient polluted atmospheres as well. At least two major differences exist between smog chamber simulations and real polluted atmospheres; first, meteorological conditions can be effectively controlled in chambers and, second, higher concentrations of reactive species (ppm rather than ppb levels) can be employed. In ambient measurements of trace pollutants and studies of their interactions the former problem is intractable; however, the latter can be overcome by exploiting the enhanced sensitivity afforded by kilometer pathlength optical systems (e.g., 1000 m vs. 40-100 m pathlengths in smog chambers), in conjunction with either IR or visible-UV methods.

Identification and Measurement of Labile Noncriteria Pollutants in Ambient Photochemical Air Pollution

Applications of FT-IR Spectroscopy--

In collaboration with Dr. P. L. Hanst, of the EPA, who designed and furnished the original instrument, a very long pathlength FT-IR facility was established at SAPRC in 1976. In this system, an FT-IR spectrometer is interfaced to a multiple-reflection cell consisting of eight gold-coated mirrors with a 22.5-m base path (Figure 4). This cell has been routinely operated in urban atmospheres at total pathlengths of 1 km or more.

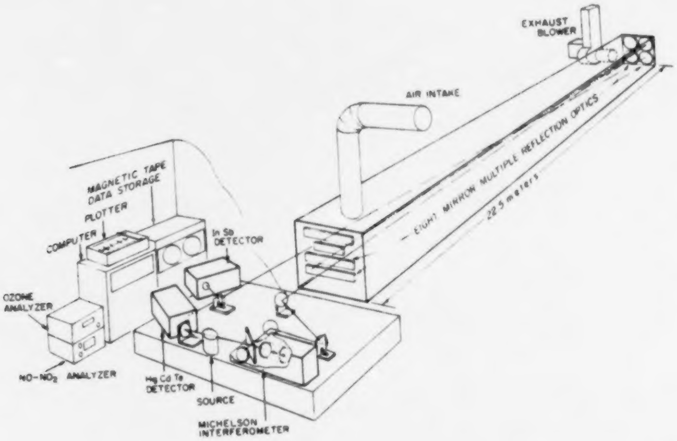

Figure 4. Kilometer pathlength multiple reflection infrared cell and FT-IR spectrometer.

During the summer of 1976, the first spectroscopic detection of HNO_3 and HCHO in ambient smog was achieved. Additionally, time-concentration profiles were obtained for these species, as well as for formic acid, ammonia, PAN and ozone (10). These measurements were carried out at Riverside, California, a "downwind receptor" site in the South Coast Air Basin.

In 1978 the system was moved "upwind" to Claremont, a mid-basin site. Again the diurnal and seasonal variations in the ambient levels of nitric acid and formaldehyde were measured concurrently with ozone, PAN, formic acid and ammonia. A representative set of data obtained in a continuous 36-hour monitoring effort on October 12 and 13, 1978 is shown in Figure 5.

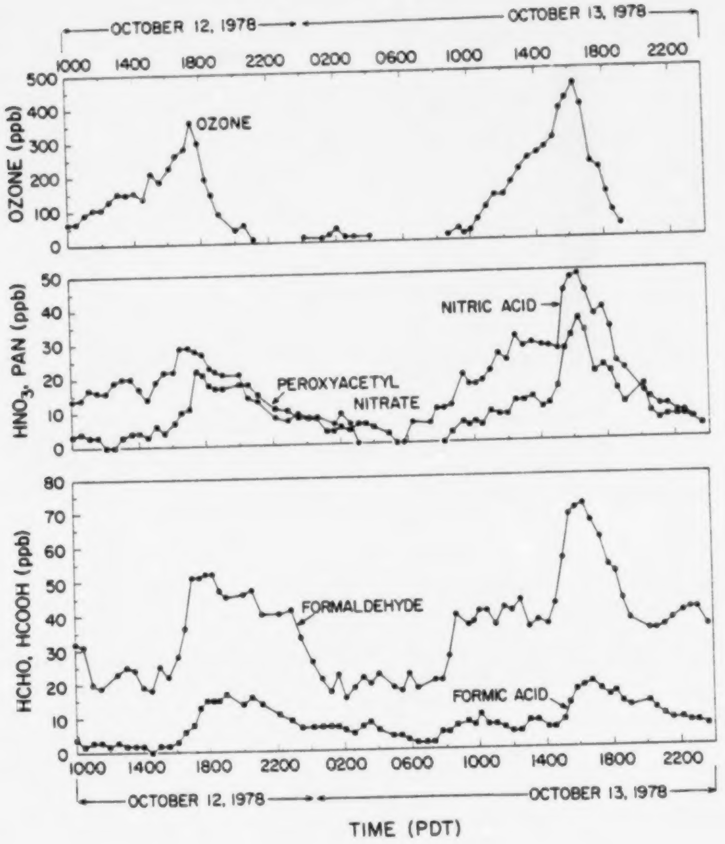

Figure 5. Time-concentration profiles of ozone and other toxic pollutants present in photochemical smog (i.e., photochemical oxidant) determined with 1 kilometer path-length, Fourier transform infrared spectroscopic system in Claremont, California during October 1978.

High "noncriteria" pollutant levels were observed during this severe smog episode. Indeed, one can see from these plots, as well as from the bar graph, Figure 6, that on this particular occasion the aggregate of these known or potentially toxic species represented a very substantial fraction of the levels of the ozone itself. For example, on the afternoon of the 13th when the ozone level was 0.12 ppm, the sum of formaldehyde, formic acid, nitric acid and PAN was 0.11 ppm. A more complete set of data from these experiments has recently been published (8).

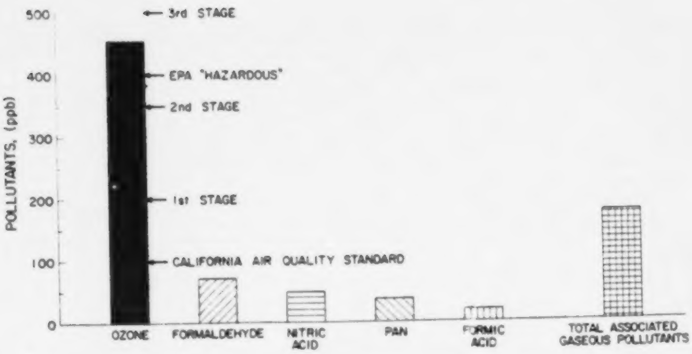

Figure 6. Maximum concentrations of ozone and "noncriteria" pollutants determined by kilometer pathlength FT-IR spectroscopy during a severe October smog episode in Claremont, California in 1978.

Applications of Long Pathlength Differential Optical Absorption Spectroscopy--

While kilometer pathlength FT-IR spectroscopy is a powerful tool for the study of atmospheric systems, it suffers from certain inherent disadvantages. Such facilities are expensive, complex and not readily mobile--i.e., not suitable for tracking pollutants across an air basin over a time span of several hours or days. Even more importantly, the IR extinction coefficients of certain key labile species are significantly smaller than the corresponding UV coefficients --resulting in a corresponding lower IR sensitivity (for a given optical path).

For these reasons, and in order to further probe the constituents of polluted atmospheres, we recently assembled and employed a long path UV-visible spectroscopic instrument to act as a complement to the km pathlength FT-IR system. The computer-based UV-visible instrument, called a long pathlength differential optical absorption spectrometer, was developed by a group of German scientists from the Institut fur Chemie, Kernforschungsanlage, Julich, directed by Professor Dieter Ehalt. These scientists first used the system (shown in Figure 7) to identify and measure formaldehyde in the sub-ppb range in maritime air using a 10 km pathlength (11). Subsequently, for the first time, they unequivocally identified nitrous acid (HONO) in a relatively clean atmosphere (12) at Julich, a small nonindustrial town in West Germany.

Last summer (1979), in collaboration with Drs. Ulrich Platt

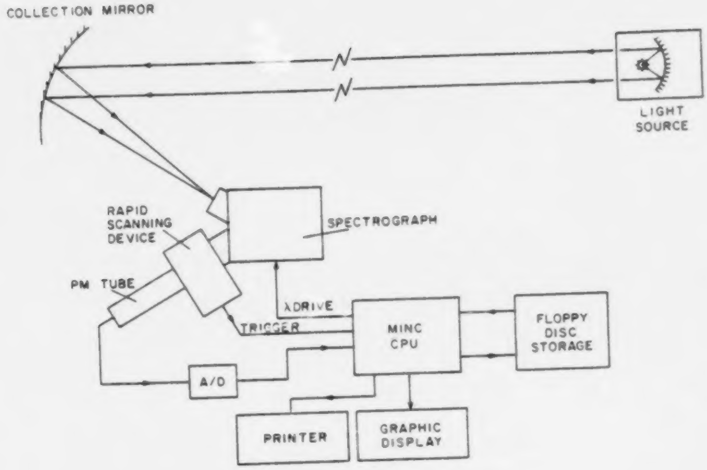

Figure 7. Schematic of SAPRC long pathlength differential ultraviolet visible spectrometer.

and D. Perner, our differential optical absorption spectrometer was employed in a single pass mode over atmospheric pathlengths of 0.5 and 1.0 km under various conditions of photochemical air pollution at Riverside and Claremont, CA. We identified and measured for the first time in photochemical smog both nitrous acid (HONO) and the nitrate radical (NO_3). Typical concentration-time profiles obtained during our study for these species are shown in Figures 8 and 9.

The details of these experiments and our results have been published elsewhere (13,14); some implications of the confirmed presence of HONO and NO_3 (long postulated to play important roles in atmospheric chemistry) in photochemical smog are briefly discussed below, with special attention to their possible interactions with POM.

REACTIONS OF BENZO(a)PYRENE WITH NITROGEN DIOXIDE AND OZONE IN SIMULATED ATMOSPHERES

Our discovery in 1975 of direct mutagenic activity of the organic extract from samples of ambient aerosol collected throughout southern California led us to investigate the reactions of benzo(a)pyrene deposited on glass fiber filters (used for hi-vol sampling) with (a) the gaseous components of ambient photochemical smog, and (b) with NO_2 and O_3 at

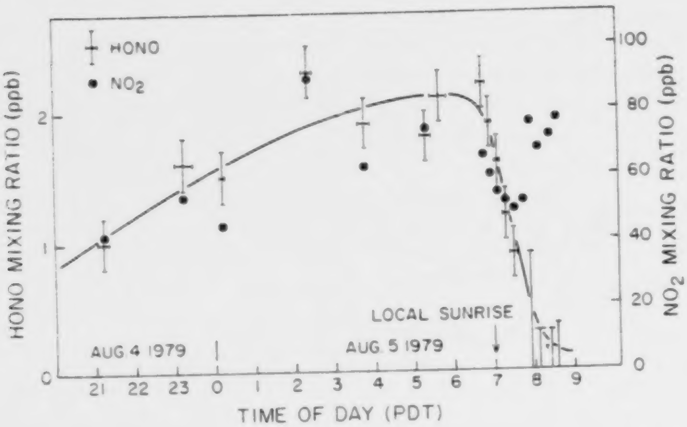

Figure 8. Concentration-time dependence of HONO and NO$_2$, measured by Differential Ultraviolet Visible Spectroscopy (DUVVS) system at Riverside, California, August 4-5, 1979.

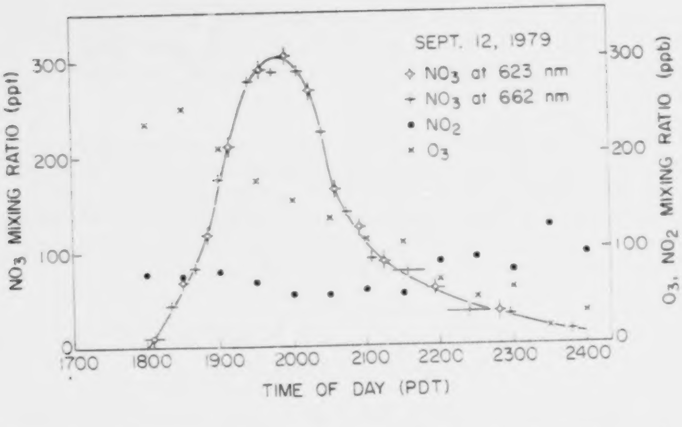

Figure 9. Concentration-time dependence of NO$_3$, NO$_2$ and O$_3$, measured by Differential Ultraviolet Visibile Spectroscopy system at Riverside, California, during a photochemical smog episode on September 12, 1979.

simulated ambient levels in the laboratory.

Extraction and fractionation of the reaction mixtures, initially by TLC and subsequently by HPLC techniques, showed that a number of derivatives of BaP were readily formed in both types of experiments. This was interesting since for many years, despite certain intriguing observations to the contrary, it was assumed by some scientists that polycyclic aromatic hydrocarbons were virtually inert in atmospheric systems.

Furthermore, in contrast to BaP, which requires microsomal activation to produce mutagenic activity in the Ames assay, directly[1] mutagenic mononitro- or oxygenated derivatives were readily formed upon exposure of the BaP-coated filters to either 0.25 ppm NO_2 (+ ~10 ppb HNO_3) or to 0.1 ppm O_3 in air, respectively. The values of 0.25 ppm NO_2 and 0.10 ppm O_3 for one hour correspond to the California air quality standards for these pollutants. The NO_2 standard is frequently exceeded in the coastal and downtown regions of Los Angeles and its immediate environs (e.g., West Los Angeles and Pasadena) in the winter months. The O_3 standard is exceeded over 200 days/year (and at times by as much as a factor of 4) in the mid-basin and "downwind" regions in the late spring, summer and fall.

It is also interesting that a directly mutagenic mononitro derivative, 3-nitroperylene, was formed when ppm levels of NO_2 (+ ~10 ppb HNO_3) in air was passed over perylene similarly deposited on a glass fiber filter. The parent PAH, perylene, is an isomer of BaP, but a much weaker activable mutagen than BaP.

We have hypothesized that such reactions with NO_2 and O_3 may, in part, account for the formation of the compounds (as yet unidentified) responsible for the "excess" carcinogenicity found in samples of POM collected from ambient air or exhaust emissions from LDMV with spark ignition engines. We use the term "excess carcinogenicity" to describe that activity which is greater than can be accounted for on the basis of the known carcinogenic polycyclic aromatic compounds measured in these samples.

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[1] We define "direct mutagen" as mutagenic activity without the use of a microsomal activation system (S9) under normal testing procedure. We are aware of the possibility of increased sensitivity of the intracellular nitrogen reductase enzymes of the original tester strains and the potential for false positives.

The half-life of BaP on a glass fiber filter exposed to 0.1 ppm O_3 in air is less than one hour; such high reactivity and the formation of direct mutagens are consistent with our earlier hypothesis that directly mutagenic and/or carcinogenic compounds can be formed by "atmospheric activation" of PAH during transport in regions with moderate to heavy photochemical air pollution. However, it is important to recognize that these exposures have been carried out on glass fiber filters. There well may be a substrate dependency of the rates and products of nitration or ozonolysis of BaP. Results of such exposure may differ significantly when carbon, quartz, Teflon fibers--or indeed raw diesel POM--serve as the sites of BaP deposition. Studies are currently underway to explore this issue.

The nitration of BaP in simulated atmospheres appears to be catalyzed by nitric acid--and as our longpath FT-IR studies have shown, there is ample HNO_3 in ambient photochemical smog to serve this function. Moreover, we now have confirmation of the presence of HONO and NO_3 in ambient air. It is possible that these co-pollutants of POM may also react with BaP and other reactive PAH.

Furthermore, the reaction of BaP with relatively low levels of O_3 (100 ppb) is so fast on glass fiber filters that this reaction would appear to be quite feasible in real atmospheres if the substrate dependence is small. To determine whether this is really the case, we must develop an "artifact free" system for collecting ambient POM. We are currently designing a "diffusive de-nuder" that selectively removes the reactive gases NO_2, PAN and O_3 prior to collection of the POM. Preliminary results at relatively low flows have been encouraging, and if substantiated for higher collection rates, may ultimately lead to an "add-on" device for particulate samplers that will minimize artifactual problems in the routine collection of ambient POM.

Finally, we point out that in order to reliably establish correlations between, for example, oxidant and NO_2 levels and the direct mutagenic activity of ambient aerosol samples, it is necessary to improve the precision and accuracy of the chemical and microbiological procedures involved in the complete characterization of an environmental sample of POM. We have refined our analytical and Ames test procedures with the goal of achieving such improvements (15). The dose-response data shown in Figure 10, are from four samples of ambient POM collected concurrently on the SAPRC "mega sampler". The good agreement between these curves suggests that overall precision for sample handling, from collection through extraction and chemical and microbiological assays, of around ±15% can be achieved if appropriate precautions are taken.

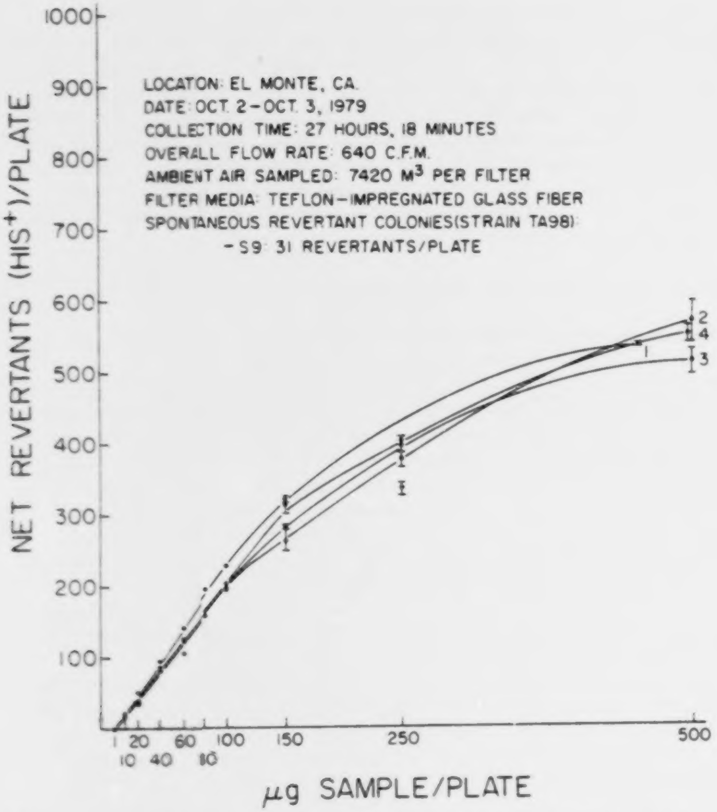

Figure 10. Direct mutagenic activity of four samples of ambient POM with strain TA98 collected concurrently using the SAPRC "mega sampler." The samples were collected on Teflon impregnated glass fiber filters at El Monte, California on October 2-3, 1979. (Spontaneous reversion frequency for TA98 was 31 revertants per plate.)

DISCUSSION OF POTENTIAL HEALTH IMPLICATIONS

We start from the premise that the composition of POM emitted from the tailpipe of light duty motor vehicles with diesel or spark ignition engines (or indeed, emitted from a fossil fueled power plant) may be very different chemically and in its biological activity from the POM that ultimately impacts man. Thus, assessments of the health effects of exhaust emissions from diesel engines must take into account not only the chemical and physical characteristics of the primary emissions of particulate organic matter, but also their chemical and physical transformations which may occur before or during receptor impact. Such transformations of POM by gaseous co-pollutants might occur: (1) in primary exhaust streams, (2) during transport in polluted atmospheres, (3) on filters during the act of collection and (4) possibly *in situ* in the lungs.

Transformations of POM in Primary Exhaust Streams

A common assumption is that if PAH levels in exhaust streams are reduced by certain control techniques (e.g., by a catalytic converter on a diesel engine operating in a mine) the health impact of the POM will necessarily be lessened. Actually, this may or may not be the case, especially for an aged and inefficient catalyst. Thus, if BaP reacts (and disappears from the system), new species may be formed which are more mutagenic and/or carcinogenic than the original BaP. If this is the case, the health impact may be worse than before treatment of the POM by an oxidizing catalyst; if not, genuine gains will be achieved.

Transformations During Transport in Polluted Atmospheres

Since diesel particulates fall in the respirable, submicron particle size range, they can remain in the atmosphere and be transported and transformed over a period of hours (or possibly even days under the stagnant weather conditions characteristic of severe smog episodes).

Three general cases should be considered when developing "impact" or "assessment" documents: injection of diesel emissions into (a) "clean" air, (b) light-to-moderate air pollution and (c) severely polluted atmospheres. In addition, two general types of "smog" that are chemically quite different should be considered. "London"-type smog contains carbonaceous particles and oxides of sulfur (SO_x) in a generally reducing atmosphere at relatively low temperatures and high humidities, while "Los Angeles"-type photochemical smog is characterized by highly oxidizing atmospheres, high ambient temperatures, clear skies and lower relative humidities.

Chemistry and health effects, of course, can be very different for diesel POM in these two quite different types of smog. We stress that our comments here are directed primarily to major air basins already suffering from moderate (~0.20 ppm maximum hourly average several times per year) to severe (>0.30 ppm O_3 hourly average on say, five or more days per year) levels of photochemical oxidant. In principle, however, the suggested approach to this problem --i.e., research on the possibility of secondary pollutants interacting in the atmosphere with diesel POM--also seems appropriate for areas with heavy sulfurous type smog.

Finally, it is clear that when evaluating the chemical and physical transformation of POM, gaseous co-pollutants to be considered should include not only the "criteria" pollutants but also such species as PAN, nitric and nitrous acids, formaldehyde, formic acid and the nitrate radical (NO_3). We have recently shown (16) that the latter reacts very rapidly with phenol and the cresol isomers, as well as with olefins; it also may do so with PAH.

Transformations of POM During Sampling

As noted previously, it is critical to define precisely what we mean by the "dose of diesel POM" and to be aware that its properties may be substantially modified by, for instance, sampling. Thus recent studies in this and other laboratories show that "filter artifacts" can occur in which, for example, reactive polycyclic aromatic hydrocarbons such as benzo(a)pyrene are transformed on conventional hi-vol glass fiber filters by gaseous pollutants present in ambient photochemical smog. They may also react with SO_x species (e.g., SO_2, SO_3, H_2SO_4, etc.), but we have not as yet examined these systems.

One of the most intriguing *preliminary* results from our studies of the interactions of ozone in air at ambient levels with BaP on glass fiber filters is that we have some evidence, based on HPLC retention times, mass and fluorescence spectra, that a BaP-epoxide may be formed. However, the analysis of the complex product mixture formed in this reaction is difficult. We can state that the HPLC fraction which shows strong direct mutagenic activity contains predominantly quinones which are inactive to strain TA98 in the Ames test. Thus, we suspect that the activities in this predominantly quinone fraction may be due to small amounts of a powerful mutagen(s) formed from BaP during its oxidation by ozone. Studies are underway to further resolve this fraction and to identify this powerful mutagen which we suspect may be an epoxide of BaP.

Should this mutagen prove to be a BaP epoxide, it is tempting to speculate that it might also be one of the species present in raw diesel exhaust and in ambient POM--and be a contributor to the strongly mutagenic activity of the polar fractions of these materials. While it is speculation to suggest that BaP-epoxide might be present in raw diesel exhaust, it is a possibility which should be investigated, since it is well known, for instance, that BaP-4,5-oxide is a very powerful direct mutagen.

In photochemical smog substantial levels of ozone coexist with BaP present in ambient POM. If the reactions on glass fiber filters do indeed occur in ambient air such a species as the epoxide may well be formed. We are currently investigating this possibility.

Transformations of POM In Situ in Lungs

If reactive PAHs--some of which are known carcinogens--react on filters with atmospheric pollutants such as nitrogen dioxide and ozone, they might also react in situ after deposition in the lung with such gaseous co-pollutants (possibly dissolved in lung fluids). Hence, even laboratory animal exposure studies designed to elucidate health effects arising from emissions of diesel POM to the atmosphere may be incomplete unless the studies simulate this potential synergism between the POM and its co-pollutants.

Implications to Epidemiological Studies

The ease with which BaP deposited on glass fiber filters reacts with ambient levels of NO_2 and O_3 to form direct mutagens has implications not only for artifacts in analyses, but also for epidemiological studies in which ambient BaP (or POM) are correlated with lung cancer. In this context, the following observation should be considered: it was reported in 1972 that levels of BaP in ambient air in California's South Coast Air Basin were much lower than in most other major cities in the world (5).

From this it could be concluded that (a) primary emissions of POM (and associated BaP) from industry and motor vehicles were much lower in the Los Angeles area than in other urban areas and (b) since BaP levels were lower in Los Angeles, the risk of lung cancer that may be associated with ambient BaP was correspondingly less. However, if the rates of reaction of BaP with such species as O_3 or NO_2 are as fast in ambient photochemical smog or during the sampling procedures employed in these studies, as we have shown them to be in our laboratory simulations, these processes could convert the BaP into species not detected by the analytical techniques commonly used in the 1950-1970 period (e.g., fluorescence). This alternative explanation could account for the relatively low levels of BaP reported for Los Angeles (5).

In this regard, epidemiologists Goldsmith and Friberg (17) report the following anomaly:

> "If urban pollution by benzo(a)pyrene makes an important contribution to the urban excess, lung cancer in the locations most polluted by this material should be highest, and when the agent decreases, lung cancer should do so as well. This has not been shown to occur."

Actually there may be little reason to expect that such a correlation should exist in areas heavily polluted with photochemical oxidant. In these regions the BaP may be efficiently transformed into other compounds in the air or on the sampling filters. Thus, the ambient levels of BaP as reported by air monitoring networks --and utilized by epidemiologists and control officials estimating health effects--may be seriously misleading.

In conclusion, atmospheric scientists should continue to attempt to define the complexities inherent in fully characterizing the atmospheric "dose" associated with diesel emissions. However, of course, the ultimate judgments concerning possible health effects (i.e., search for and characterization of the "response") must be made by the biological and medical community--hopefully in close collaboration with the atmospheric scientists.

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General Discussion

R. KLIMISCH: There is some work that says that BaP isn't nearly as reactive when it is on soot. It doesn't react with NO_2 very readily, and I would suspect that it won't react with anything else very readily when it is absorbed on soot. So I really think your experiments are unrealistic in terms of the reactions of PAH.

J. PITTS: That's a very good point and a very good question. You recall I stressed that it might very well be substrate dependent. This may very well be the case. We also have received, in the last six months, a grant from the Department of Energy to look at exactly this question. What are the reactions on glass fiber, on soot, on coal fired power plants flyash. They will likely be very different. Certainly, I think the photochemistry is different, and I have not even talked about just direct photochemical rearrangements. There is evidence in the literature that suggests the photochemistry just in clean air may be very different on, say, flyash as against soot. So your point is well taken, they may not, but we are going to continue in a very orderly fashion to try to explore these different subjects. We have an application in now and hopefully, we will be working with DOE and NSF to use photoacoustic photoscopy to actually get the absorption spectrum of these PAH's and PNA's as they are bound on these different substrates. As an old geriatric photochemist who has been in this game probably longer than he wants to admit, the first thing we taught our students, and the first thing I was taught, is before you start any photochemical studies, you have to measure the absorption spectrum for the compounds you are looking at. The first law of photochemistry says if it doesn't absorb, it isn't going to react photochemically. Well, very little is known about absorption spectrum of PAH's. So this is going to be a very exciting field, and it is a tough field, but it is exciting and it will provide answers to all kind of questions.

R. KLIMISCH: I think you will find that PAH's are always associated with particulates and carbon,

J. PITTS: Depending on whether they are sitting on the flyash inorganic site with a particular service area and service density, and service properties as against say the kind of loose soot that are hung together in diesel, they can be very different.

B. BELINKY: With respect to the nitro derivatries of PAH, have you actually determined their presence in either diesel emissions or ambient air, and have you received any comments about their susceptibility.

J. PITTS: We have not as yet. We have looked for nitros at Riverside but one problem is that NO_2 levels are very low at that location. We never exceed the air quality standard there. We will be looking for nitro derivatives this winter near the beach in Southern California where NO_2 levels are high. The second problem is that they are photochemically reactive. We have not measured the quantum levels as we intend to, but they do photo decompose and ultimately wind up as clinodes. The NO_2 rearranges to a nitrate to NO bond, NO rips off in sunlight and then you oxidize it ultiamtely to a clinode. Here is another point which should be mentioned. In these transformations, I want to be very clear about this, we may form compounds that are more toxic. We may also detoxify the compound. It is very important to recognize that if you allow ozone oxidation to proceed long enough, you will have a system comparable to the S9 oxidation system cells. That is, the PAH's are oxidized with formation of hydroxyl radicals and thus might be detoxified also. We have to look at that path.

J. PATTISON: I presume the nitrate radicals were measured in Riverside. However, this might have been the time of day that the LA smog drifted into that area, rather than say that it is forming at that time of day which was late. So your interpretation is, if you measured it downtown you would find it in the day rather than in the early evenings?

J. PITTS: That is a "downwind" phenomena. Most of the smog comes into Riverside, for example, from LA along the mountains, Pasadena, Ontario, up through Oregon County. So this would basically be it.

J. PATTISON: In other words, it drifted in rather than formed there.

J. PITTS: It drifted in, but it just sat there. It sat there lone enough - it was a fairly stable mass.
It had moved in and, in fact, it was an episode that lasted several days. We never went down to clean air. We didn't have a transport through to clean it up every night.
This was like that Clairmont episode. It stayed, and it built up, so the inversional phenomena of the build-up during the night is a real phenomena.

J. PATTISON: In other words, it would be retained in the evening?

J. PITTS: No, no, in this case the nitro derivatives formed and when the sun went down disappeared. So it was formed by photochemistry, but it didn't get there sooner because it hadn't drifted in yet. It had to be actually made there. It was in the element of the smog mask that moved in - contained in certain compounds then when the right NO conditions, particularly concentration and ratio were reached, it could form which it did. Then with the addition of NO, NO_x reacts with NO, NO_3 radical plus NO reacts at collison frequency practically to form NO_2. So it is destroyed. If you have a lot of cars go by the freeway, you get more NO, and it is gone. It is a very complex system, but it is there.

OPTIMIZING DIESEL COMBUSTION: IMPROVING FUEL ECONOMY, ENGINE LIFE, AND REDUCING PARTICULATE AND NO_x EMISSIONS WITH ELECTROSTATIC FLUID PROCESSORS

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ABSTRACT

Emission legislation has adversely affected fuel economy of gasoline engines, and threatens similar effect upon diesel-powered vehicles. This would be unfortunate, for diesel automobiles can have a significant fuel conservation impact, improving fuel economies by as much as 100 percent over comparable gasoline engines in actual use-modes.

Increasing application of diesel power to autos and light trucks has been somewhat inhibited by two related emissions concerns: speculative concern about health effects of components of diesel particulates, and technical difficulty of simultaneous achievement of proposed particulate and nitrogen oxides emission levels.

This paper illustrates the positive potential of Electrostatic Fluid Processing systems (EFP) to substanitally reduce both particulate and NO_x emissions without a fuel economy penalty, and, with added benefit of extending engine/component life. Also, as diesel fuels continue to deteriorate in quality, and as manufacturers are under

pressure from emissions proposals to develop increasingly sensitive microprocessor-controlled fuel injection equipment and/or such wear-inducing techniques as exhaust-gas recirculation, the role of EFP is expected to assume critical importance in diesel power applications.

INTRODUCTION

Relatively neglected areas of inquiry in the concern about maximizing fuel economy and minimizing engine emissions are those of fuel composition, fuel quality, contamination of fuels in processing, handling and distribution, and in fuel systems, and, related issues regarding interaction of lubricating oil contamination, engine emissions, engine life and fuel economy.

Obviously, concern with emissions from fuel burned in an engine or other combustion system properly includes concern and inquiry about the incombustible, partially combustible, and other fuel components and contaminants which inhibit optimal combustion, and are emitted as exhaust components. The computer industry truism, "...garbage in...garbage out", is also true of fuels/engines systems.

Furthermore, in reciprocating piston engines, especially in diesels, crankcase lube-oil contaminants may plate upon or even aspirate into combustion chambers, and there intermingle with fuel and fuel contaminants, causing an increase in emission product.

Such contaminants, in addition to the partially-understood phenomenon of "carbonizing" (1), dynamically affect combustion in the known and partially known phenomena of wall-quenching, flame quenching, and fuel droplet-quenching. Such contaminants may also act as "condensation nuclei" and chemical reactants within the fluid dynamic of combustion.

It is known conclusively that automotive fuel filters and lube-oil filters of nominal 5-micron size capacity actually cannot remove particles smaller than 10 to 13-microns with consistency. It may be confidently asserted that fuels and lube oils, as they are used in engines, have contaminants, residues and detritus which are larger and much smaller than these sizes. These contaminants contribute to engine wear, to reduced fuel economy, and to increased emissions. Conversely, removal of and/or prevention of the formation of such particulates as appear in fuels and lube-oils of diesels will have a salutary and economical effect upon the parameters of emissions, fuel economy and durability.

TECHNIQUES OF PARTICULATE REMOVAL FROM HYDROCARBON LIQUIDS

The problem of removing very fine particles from a body of fluid is not readily solved by mechanical filter alone, since it becomes necessary to use a filter element having such fine pores or openings to pass the fluid that they are easily clogged by removed particles lodged in the pores. The resistance to flow increases rapidly as the pores become clogged. Resistance to fluid flow would be intolerably great even without such clogging when the openings are sufficiently small to retain particulates of micron size.

Centrifuges (centrifugal separators) remove particles and particulate contamination to the 10 micron range, and less consistently to 5+ micron size. It is interesting to note the effect of even this relatively poor level of diesel fuel purification upon parameters of contamination and combustibility:

TABLE 1. BENEFITS OF PURIFICATION
WITH CENTRIFUGE EQUIPMENT (2)

	Before Purification	After Purification
Ash Content	0.09%	none measurable
Water Content	1.3 %	none measurable
Net Calorific Value, BTU/lbs.	17,603	18,081

Particulates of 10 micron are the smallest size visible to the unaided eye (2), and, according to ASME/ASTM Standard 118, oil purification equipment must remove particulate at least to 10 micron. Other specifications call for reduction of particle contaminants to one micron size.

Water-washing, a technique which removes particulate from fuels and oils to one micron, but not smaller, is highly unlikely to find application aboard a motor vehicle.

However, since hydrocarbon liquids are dielectric fluids having relatively high dielectric value at a wide range of temperatures, an electrostatic field may be employed to remove particles down to 0.001 microns, (10^{-9} meter) (3).

THE ELECTROSTATIC FLUID PROCESSING SYSTEM (EFP)

It is well known, especially in treating dirty gases, that suspended particles can be charged electrically and then caused to migrate to and be collected on a surface under the influence of an electric field. In a typical electrostatic precipitator, the force of the electric field on a 0.5 micron (micrometer) particle is several thousand times the force of gravity on such a particle. When hydrocarbon liquids are substantially free of water, the force of an electric field upon particles in such a liquid hydrocarbon remain approximately the same as that upon particles suspended in a gas.

The EFP system was originally designed for cleansing fluids for missiles, where there is a definite need for liquid of the greatest possible freedom from contamination, and the liquid to be cleaned has a high dielectric value. EFP systems have been used successfully for many years in such applications. It is a system that will remove very fine suspended particles, and includes an effective water-stripping component capable of removing water to 0.005%. The system is an electrostatic processor capable of removing sub-micronic particles from a dielectric liquid stream or bulk storage.

The EFP unit designs offer negligible resistance to fluid flow. It is flexible in operation, economical to manufacture in various capacities and flow rates, and is simple to maintain in efficient operating condition. EFP systems remove macro and sub-micronic particulates from fuels and lube-oils, and remove liquid contaminants by breaking up emulsions.

EFP SYSTEM PRINCIPLES DIFFER FROM GASEOUS PRECIPITATORS

While the EFP system uses electrical charging fields, principles of dielectrics, electrostatics and electromagnetics similar to those used in electrostatic gaseous precipitators, the phenomena of corona discharge used in gaseous precipitators do not occur in dielectric liquids. Rather, electrostatic induction, electrophoresis, and/or electrowinning agglomeration processes are used to remove all particulate contaminants without destroying any of the liquid or changing any of its chemical or physical properties, except that removal of contaminants restores the original viscosity, improves flamability and the like.

A high voltage electric charge, applied to the fluid causes an excess of electrons in the fluid. These electrons separate covalently bonded materials by filling the void in the valence of the co-valently bonded atoms. By this means, dissimilar materials are separated and similar atoms then ionize, flocking together into larger particles which adhere to the charged surface of the opposite polarity. Thus, elemental as well as chemically complex substances, whether metallic, non-metallic, organic or inorganic, can be removed from petroleum liquids and non-petroleum hydrocarbon liquids.

EXPERIMENTAL DETERMINATION OF SUBSTANCES REMOVED

Test conducted by the U.S. Air Force over a period of several years determined the following substances are effectively removed by EFP systems processing fuel oils, hydraulic fluids, turbine oils and lubricating oils (4):

Iron
Aluminum oxide
Red iron oxide
Biotite mica
Limestone
Titanium
Manganese
Lead
Silver
Zinc
Steel
Quartz
Delustered dacron
Brass, bronze
Calcite
Hornblende
Calcium
Silicon
Aluminum
Chromium
Phosphorus
Chlorine
Stainless steel
Magnesium
Limonite
Paper fibers
Carbon
Potassium
Copper
Tin

PROLONGED ELECTROSTATIC PROCESSING EFFECTS ON HYDROCARBON LIQUIDS

The U.S. Air Force subjected used purging oil to 80 hours of electrostatic filtration, with the following test results:

Sample	Flash Point
New Purging Oil	208° F
Used Purging Oil Before Filtration	151° F
Used PUrging Oil After 80 hrs EFP Filtration	162° F

	Viscosity
Used Purging Oil Before Filtration	.0516 centistokes
Used Purging Oil After 12 min.	.0526 centistokes
Used Purging Oil After 8 hrs Filtration	.0526 centistokes
Used Purging Oil After 60 hrs Filtration	.0524 centistokes

INFRARED ANALYSIS

Samples of used purging oil were compared with the used purging oil after it had been electrostatically filtered for 80 hours and no change in the composition was detected (5), other than the removal of contaminants, which tends to improve viscosity, etc. The Air Force laboratory investigators concluded:

> "We have not encountered any type of contamination that is not effectively removed by the electrostatic filters. Contamination commonly found in hydraulic systems and motor oils is effectively removed...The fact is, we have not found any kind of solid material the filter will not remove, regardless of how small it is. The electrostatic filter removed particles of 5 microns and smaller very effectively." (Whereas) ..."Mechanical means of filtering using 5-micron absolute filters, cannot remove this contamination and the level of this small size, 5-micron and smaller, would gradually increase, causing changes in the viscosity of the fluid...The smaller the particle the more effective is electrostatic filtration..." (6) (Emphasis in original).

ON-BOARD ELECTROSTATIC PROCESSING OF DIESEL FUEL AND DIESEL ENGINE LUBRICATING OIL -- EXPERIENCE IN THE MEXICAN NATIONAL RAILROAD

The popular press in the United States has characterized Mexico as a nation unconcerned with environmental pollution, and since revelation of the extent of Mexico's petroleum reserves, as also unconcerned with conservation. As will be seen, this is unfair to Mexico and is in fact, not the case.

The Mexican National Railroad and the Mexican government have expressed their interest in conservation of fossil fuels and in reducing pollution from combustion by having sponsored a three-month test of EFP units aboard an Electro-Motive Division (EMD) diesel locomotive of the Ferrocarriles Nacionales de Mexico. From the results of this test, the Mexican government has expressed its interest in installation of EFP systems on all 1,500 Mexican railroad locomotives.

SUMMARY OF RAILROAD DIESEL TEST RESULTS

An EFP system consisting of two EFP units 27 inches wide by 46 inches high by 36 inches deep was installed in an

EMD SD40 3000 HP diesel locomotive for continuous processing of engine fuel and lubricating oil. Units were use-dedicated, one for diesel fuel and one for lubricating oil, and were installed aboard Engine #8720 in Mexico City. Another locomotive, Engine #8709, also an EMD SD40 3000 HP model, was used as the control or comparison engine. Both engines had just received major overhaul, and were as close to being mechanically identical as was possible. The two engines were coupled together to permit them to receive identical fuel, lubricants, filters and operators. They were designated to pull the same load under identical conditions. The only significant difference between the two engines was the EFP system aboard #8720, processing the fuel and lube-oil of #8720's diesel engine.

1. One of the major differences in operation noted was the disappearance of the majority of the black smoke emitted by locomotive #8720, which contained the EFP system.

2. Another significant change was the cleanliness of fuel as it passed through the sight-glass. Upon installation, the fuel passing the sight-glass was extremely dark and non-transparent. Within minutes after the EFP system was turned on, fuel passing the sight-glass changed to clear and lighter color.

3. Fuel tanks on locomotives apparently have tendency to build up an iron oxide scaling, extremely small in micron size, which is continuously carried through to injectors and engine combustion chambers by the fuel flow. After the EFP system was operating, the excessive fuel over engine demand was force-fed back to the fuel tanks. Iron oxides which were stripped from the inner tank walls were captured and retained by the system, preventing these contaminants from being transmitted to the injectors, combustion chambers, and hence, to exhaust emissions.

4. With regard to fuel cleanliness, the locomotive tanks were filled with centrifuged fuel, which purportedly is free of contamination by particulate matter and water. A sample of the fuel was taken while the tanks were being filled. Then the locomotive was started and the EFP system turned on. At the end of three hours, a sample of processed fuel was taken from the drain of the locomotive tank. Both samples were in the same size and type containers; the difference was so visible that the centrifuged fuel appeared unprocessed when compared to that processed by EFP.

5. The incandescent material normally emitted from the stacks of a diesel locomotive began to visibly disappear

from the emission of #8720, while it increased in that of #8709. Within three days, the incandescent emission from #8720 had been reduced to the extent that it was completely cooled and no longer visible beyond a two-foot distance of the stack. The possibility of #8720 starting a roadside fire had been eliminated, and the total mass particulate emissions had been dramatically reduced as evidenced by substantial reduction of smoke and other visible particulate emissions. Most certainly there was a reduction of particles of iron oxide which are normally emitted from the locomotive's exhaust stacks.

6. Upon termination of the test, examination of the cathodes revealed the EFP units had captured and retained various contaminants ranging in size from the smallest visible under a microscope to sizes in excess of 100 microns. While we have no calculation of the percentage this removal is of the total fuel mass consumed in the test, there were 10 collecting plates of approximately 16 inches in diameter that contained a coating in excess of 1/8-inch thickness of contaminants removed from the fuel. Further examination of the polyurethane pads of the EFP unit, and the fuel residue in the bottom of the EFP housing revealed considerably more of the same sizes and types of contaminants. A good portion of the samples were given to Mexican Railroad chemists, therefore it was not possible to estimate a ratio of fuel/contamination or the percentage of contaminants in the fuel.

7. Within one week of operation (7 days), the engineer complained of the difference in performance between the two locomotives. He felt the fuel was being restricted in #8709 and asked that the mechanical filters be changed. The mechanical filters in both locomotives, #8709 and #8720 (EFP-equipped), were changed simultaneously. The weights of the filters prior to and after installation were recorded:

TABLE 2. WEIGHTS OF MECHANICAL FILTERS ON LOCOMOTIVES

	Primary	Secondary #1	Secondary #2
New Filters (dry)	2788 g	230 g	230 g
New Filters (wet)	3895 g	320 g	320 g
#8709 Wt. on removal	4056 g	330 g	326 g
Percent Wt. increase over wet	3.97 %	3.03 %	1.84 %
#8720 Wt. on removal	3895 g	320 g	320 g
Percent Wt. increase over wet	0.0 %	0.0 %	0.0 %

Thus, it may be seen in Table 2, that on-board, in-use application of EFP systems processing of diesel fuel prevents a significant percentage by weight, of contaminants from reaching the standard mechanical filters. It may be confidently asserted, therefore, that EFP systems prevent a significant percentage of contaminants smaller than 5 microns which would usually pass through conventional filters, from reaching combustion and from being emitted as exhaust product.

FUEL ECONOMY IMPROVEMENTS

The first distance run made by the locomotives was a three hundred (300 km) kilometer round trip. Upon their return, they were refueled and the quantities recorded:

TABLE 3. FUEL CONSUMED, AMPERAGE DEVELOPED

Locomotive	Fuel Consumed
#8709	2069 liters
#8720 (EFP-equipped)	1862 liters
Difference:	207 liters = 10 percent
	Amperage Developed/Traction Motor Load
#8709	500 Amperes
#8720	500 Amperes
	Inches of Rack Indicated for 500 Amp Load
#8709	92 inches
#8720	104 inches
Difference:	12 inches

(Note: The lower the inches of rack the more fuel consumed.)

When two locomotives, coupled back to back, are pulling the same train and the inches of rack are at different settings to produce the same amperage loading, the engine with the lower numerical inches of rack setting is consuming more fuel. In this case, the non-EFP equipped engine #8709 consumed 10 percent more fuel.

LUBRICATING OIL PROCESSING

The lubricant used in #8720 had no additives and was continuously processed by the on-board EFP system's lube-side unit, removing all metallic particles normally caused by wear, in addition to all of the carbon and metal-infused carbon particles, plus other inorganic substances usually found in the carter (pan) of the locomotive diesel engine.

Simultaneously, the moisture was removed from the lube-oil, preventing the possible formation of hydrochloric and sulfuric acids which have a tendency to etch the metal surfaces of engine and components. (Note: Additives used in lubricating oils for the detection of water are abrasive, and although smaller than 5 microns in physical size, cause internal wear of the engine.)

Through systematic processing of the lubricant, noticeable increase in lubricity of the fluid, combined with increased volatility of EFP-cleaned fuel, significantly and noticeably lowered the noise level of diesel engine #8720 compared to that of engine #8709. Operator comments were: a) Locomotive #8720 operates more smoothly and has more positive control, particularly when switching cars in the yard; b) The diesel engine noise is reduced from what it was before; c) The black smoke and sparks have almost completely disappeared, compared to the emissions from the sister locomotive #8709 which did not have an EFP system installed (7).

DISCUSSION OF MEXICAN RAILROAD DIESEL TESTS OF EFP SYSTEMS

While the objective results reported are impressive -- a ten percent fuel economy improvement; substantial reduction of emissions; evidence of potential extended engine life; and the fact that EFP systems can operate successfully in harsh conditions for at least 1120 hours without downtime or maintenance, the fuel economy data are from one test situation, therefore the developer does not claim a similar percent improvement will always prevail. However, the data indicate that EFP systems can provide emissions control at some level of improvement, without increasing engine frictional or thermal loadings, and without a fuel consumption penalty. This is more than an be said of many technologies being investigated for improvement of diesel combustion.

Also, while reduction of black smoke and visible particles in exhaust emission is not conclusive evidence of reduction of smaller, invisible particulate emission, the fact that incombustible particulate of sizes down to 0.001 microns are consistently removed from fuels and lube-oil prior to combustion is such evidence, and means that these very fine particulates are prevented from entering combustion, and hence, are prevented from emission. It can be said that EFP systems do reduce total particulate emission.

NITROGEN OXIDE EMISSIONS AND EFP SYSTEMS

It is known that high values of NO_x formation occur under conditions of high smoke formation (8). These conditions are a function of high temperature, heat transfer due to carbon formation and proximity to a cold surface (wall-quenching). While NO_x emissions from EFP-fitted diesels have yet to be measured, it is likely that EFP-caused reduction in smoke formation conditions is accompanied by some reduction in NO_x.

Also, to the extent that fuel volatility is increased by EFP fuel processing, combustion temperatures can be lowered, and duration of combustion may be shortened.

Secondly, EFP removal of incombustible particles from the fuel probably has the effect of reducing droplet, flame, and wall-quenching phenomena, which would tend to increase the critical concentration of combustible fuel droplets during the mean period of major heat release. If so, this would help "...win the race against the NO(x) kinetic", in the words of the distinguished Dr. W. T. Lyn, and would contribute to such victory over NO_x as is now being sought by means of increasing injection pressures and rates (9).

PARTICULATE EMISSIONS AND EFP SYSTEMS

We have seen that EFP-fitted diesels emit fewer large particles, and we have seen EFP systems prevent much of the sub-micronic particulate matter from entering combustion or appearing in exhaust emissions. This improvement occurs in diesels using fuel of relatively good quality -- a centrifuged fuel. To the extent that fuels contain carbonaceous particles in sizes 10^{-9} meter and larger, including those containing the high molecular weight ring compounds, they are removed. Certainly carbonaceous particles are formed instantaneously in diesel combustion no matter how pure the fuel and lube-oil in the engine, and we make no claim EFP will prevent all of this.

However, it is likely that much of the carbon formation in high-pressure, high-temperature combustion is stimulated by presence of contaminants and carbon residue particles of 0.001 microns and larger. Given the lack of a precise understanding of the nature of such carbon formation, a hypothesis of "precursor" particles or of "precursor electrical charges" from such particulate as is present in fuels unprocessed by EFP system should not be dismissed without empirical investigation.

In any case, EFP systems provide an absolute improvement in particulate emissions regardless of any other parameter of engine design, performance, operating conditions or fuel composition. As certainly, EFP systems will provide some level of reduction in particulate emissions additional to any provided by engine design or injection system design improvements or exhaust after-treatment.

DIESEL FUEL COMPOSITION, PARTICULATES AND EFP: MYTHS ABOUT FUEL

ASTM Limiting Specifications for No. 2-D diesel fuel allow the following amounts of solid and liquid contaminants:

Water and Sediment, % vol.	0.05
Carbon Residue, %	0.35
Ash, % wt.	0.01
Sulfur, % wt.	0.50

Except for ash and sulfur which remain the same, No. 1-D specifications are about one-half this amount of contaminant. Since EFP systems remove water to 0.005%, and consistently remove all of the above solid contaminants to the 0.001 micron level, these specifications, often not realized in the 'real world', can be surpassed by application of EFP systems.

The vastly complex subject of petroleum fuels production and refinement is beyond the scope of this paper, nevertheless, it is useful to note recent trends indicating possible decline in fuel quality, and to reflect on the ways fuels become contaminanted.

Generally, 'straight-run' diesel fuels are the highest quality, i.e., those boiled off at the lowest distillation temperature for that strata of the barrel of crude. It is said that European diesel fuel is in greater proportion, straight-run fuel, and perhaps it is not a coincidence that actual Cetane Number values in Europe are reported to be 55 CN, whereas in the United States, where more diesel fuel is derived through thermal cracking and other high-temperature, high-pressure processes, the CN average is falling to 45 CN (10).

While refineries take great care to ensure their fuels meet specifications, it would be interesting to have results of carbon residue and other contaminant averages in fuels actually available in the United States. For, it is said the demand upon refineries to produce unleaded gasoline has taken much mid-distillate stock for this purpose, requiring increased cracking of heavier stock to meet demand for diesel. Despite this, U.S. government analysis of the quality of diesel fuel is apparently still conducted upon mainly straight-run fuel samples of the highest purity available from refiner's diesel stock. If increased cracking processes are used, it would be expected that carbon residue, ash, sediments and perhaps other contaminants are increased.

Diesel Automotible Association motorist members report marked increase in water and sediment contamination of fuels as a consequence of disruption in the supply system this year. The appetite of diesel fuel for water is well known, and diesel vehicles accumulate condensation water in fuel. Increased tendency of users to store diesel fuel against threatened shortages of supply increases likely contamination of water, bacteria and fungi.

In handling, delivery of fuels and filling of vehicles, there are opportunities for contaminants to enter the fuel. High-speed flow in fill-lines generates electrostatic charges and frictional heat. Some additives, such as surfactants, increase susceptibility of fuels to water attraction, contamination and deterioration. If benefits from EFP of fuel economy, reduced emissions, reduced wear were not sufficient to attract manufacturers to EFP, insuring diesel users against further deterioration in fuel quality ought to be.

OTHER APPLICATIONS OF EFP SYSTEMS IN FUELS/OILS PROCESSING

Today we are concerned with transportation diesel applications of EFP systems, and our developers are engaged in production of EFP prototypes suitable in size and manufacturability to diesel automotive and automobile applications, however, there are several others of interest to conservation of fuels and reduction of atmospheric and environmental pollution:

- Purification of Power Plant Boiler Fuels -- especially in reduction of particle sulfur and particulate in residual fuel of declining quality.
- Purification of Combustion Turbine Fuels -- especially removal of trace metals in particle or compound form, which, when not removed, cause costly damage to turbine blading.
- Reclamation of Used Automotive Lube Oils/Cutting Oils.

- Processing Coal-Derived Liquid Fuels -- especially improving stability of coal liquid product.

SUMMARY

In addition to improved fuel economy in diesels, reduction of emissions, reduction of wear, downtime, and associated maintenance, all of which are consistent benefits of EFP systems, the following specific benefits obtain from EFP application to diesels:

- Reduced Fuel Pump/Injector Wear;
- Reduced Failure of Seals, Gaskets, O-Rings, and Polyurethane Fuel Injection Parts;
- Reduced Wear of Cylinder Liners, Piston Rings, Valves;
- Reduced Corrosion, Reduced Acid Formation;
- Improved Combustion;
- Reduced Noise;
- Reduced Carbon Deposition on Exhaust Valves, Combustion Surfaces;
- Easier Cold Starts; --

and the potential for design of EFP systems which completely eliminate oil drain intervals.

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8. "Optimization of Diesel Combustion Research", W. T. Lyn, Cummins Engine Co., the Horning Memorial Award Lecture, Society of Automotive Engineers Paper #SP-433, 1978.

9. Ibid., p. 5.

10. Diesel Automobile Association interviews of DOE and other U.S. government fuel specialists.

SESSION I

Summary Discussion Following Completion of Session I

General Discussion

A. KOLBER: Is there any information on pollutants that are produced by diesel which have a high vapor pressure or vapor phase materials that might be trapped and tested? Is there any information at all as to what they might be, and if they exist can they be tested?

J. PITTS: The ones we are most concerned with are NO_2 and NO. These levels are relatively high, in relation to the spark-ignition engine versus a diesel system. However, there are others, including some of those that he had on his slide, for example, formaldehyde. Who would like to answer that question? Someone who has actually measured the gaseous pollutants.

R. BRADOW: I guess in terms of vapor-phase components, diesel engines in general don't look greatly different from what we have seen from gasoline engines. There are a number of papers in the literature over a long period of time relative to the important gaseous constitutents of both gasoline and diesel engine exhaust. Organic compounds we are dealing with are, of course, mainly hydrocarbons. In general, about ten percent of what we measure as total hydrocarbon is present as low molecular weight oyxgenates both in gasoline and diesel engine exhausts, and these are dominated by formaldehyde. I would estimate for both gasoline and diesel engine exhaust, probably, formaldehyde would exceed all the other aldehydes combined in concentration. There are great differences in emission rates for oxygenates and hydrocarbon depending on the way the vehicle or test engine is operated. Some of the problems in making point-to-point comparisons between heavy duty diesel engines and those for the use of passenger cars, for example, are centered around the fact that it is hard to get comparable cyclic emission tests right now. Possibly when the coming transient test for diesel engines is run for these pollutants we can do a little better job in making one-to-one comparisons between types of engines. I think that so far, we have come to the conclusion that the organic materials associated with the particles, oxygenates, and hydrocarbons have molecular weight aromatic hydrocarbons and differ markedly from engine to engine. It is a little difficult to say where the sort of unifying similarities might be. I think the slides I showed this morning relative to the HPLC chromatograms for the variety of different diesel and gasoline engines were sort of indicative of that. Frankly, I would really like to have somebody else speak on that issue. Does anyone else believe that the differences among engines, diesel, and gasoline are bigger than the similarities?

R. KLIMISCH: There is one difference I would like to mention. That is the fact that evaporative emissions from diesel engines were much lower than gasoline engines. That becomes significant in terms of the total hydrocarbons.

R. BRADOW: I quess that is true. Evaporative loss from diesel engines is very low. We have seen, though, relatively low evaporative loss in a lot of newer gasoline engine cars. The important issue here however, is really tailpipe emissions. Previously when there were a lot of cars burning leaded gasoline you really had something in the evaporative loss to talk about, for example, ethylene dichloride, ethylene dibromide, and perhaps some of the lead alkyls as possible toxic agents that might be present in the ambient air to some respectable level. Evaporative emissions generally do not appear to be such a big problem any more. Also there are very few cars being made that burn leaded gasoline. I think in the 1980 model year one gasoline automobile was certified for leaded gasoline out of all the makes and models that exist. So, it appears to me that certianly in terms of oxygen precursors, we still have a little problem with evaporative loss. But we never have had the problem with regard to toxic agents that we have with tailpipe emissions. We have not tested enough different kinds of cars and do not possess the analytical techniques that are needed to assess their difference and similarities. I think these are the major issues that need to be addressed.

Session II

IN-VITRO CARCINOGENIC AND MUTAGENIC EFFECTS OF DIESEL EMISSIONS AND DIESEL EMISSION COMPONENTS

Chairmen:

Dr. Joellen Huisingh
Dr. F. Bernard Daniel

Diesel Particulate Collection for Biological Testing: Comparison of Electrostatic Precipitation and Filtration.
Chan, T. L., P. S. Lee, and J. S. Siak.

Diesel Particulate Extracts in Bacterial Test Systems.
Siak, J. S., T. L. Chan, and P. S. Lee.

Mutagenic Activity of Diesel Emission Particulate Extracts and Isolation of the Mutagenic Fractions.
Choudhury, Dilip R. and Charles O. Doudney.

Mutagenicity Studies on Diesel Particles and Particulate Extracts.
Loprieno, N., F. DeLorenzo, G. M. Cornetti, and G. Biaggini.

The Mutagenicity of Diesel Exhaust Exposed to Smog Chamber Conditions as Shown by Salmonella Typhimurium.
Claxton, Larry, and H. M. Barnes.

Salmonella/Microsome Mutagenicity Assays of Exhaust From Diesel and Gasoline Powered Motor Vehicles.
Lofroth, Goran.

Session II

(Continued)

Biological Availability of Mutagenic Chemicals Associated with Diesel Exhaust Particles.
Brooks, A. L., R. K. Wolff, R. E. Royer, C. R. Clark, A. Sanchez, and R. O. McClellan.

Diesel Particulate Matter Chemical and Biological Assays.
Risby, T. H., R. E. Yasbin, and S. S. Lestz.

Diesel Particulate Extracts in Cultured Mammalian Cells.
Rudd, Colette J.

Diesel Soot: Mutation Measurements in Bacterial and Human Cells.
Liber, H. L., B. M. Andon, R. A. Hites, and W. G. Thilly.

Studies on the Effects of Diesel Particulate on Normal and Xeroderma Pigmentosum Cells.
McCormick, J. Justin, Roselyn M. Zator, Beverly B. DaGue, and Veronica M. Maher.

Benzo(A)pyrene Alters Lung Collagen Synthesis in Organ Culture.
Bhatnaga, R. S. and M. Z. Hussain.

Application of a Battery of Short Term Mutagenesis and Carcinogenesis Bioassays to the Evaluation of Soluble Organics from Diesel Particulates.
Huisingh, Joellen, Stephen Nesnow, Ronald Bradow and Michael Waters.

A Review of In-Vitro Testing Systems Applicable to Diesel Health Effects Research.
Whitmyre, Gary K.

The DNA Damage Activity (DDA) Assay and its Application to River Waters and Diesel Exhausts.
Doudney, Charles O., Mary A. Franke, and Charles N. Rinaldi.

DIESEL PARTICULATE COLLECTION FOR BIOLOGICAL TESTING: COMPARISON OF ELECTROSTATIC PRECIPITATION AND FILTRATION

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Biomedical Science Department
General Motors Research Laboratories
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ABSTRACT

The extent to which a particle collection method can influence the chemical composition and biological activity of diesel particulate extracts was investigated. Undiluted diesel particles were collected from the exhaust of a 5.7L GM diesel engine at specific collection temperatures by electrostatic precipitation (ESP) and filtration. Parallel samples were taken with an electrostatic precipitator and Pallflex filters under the same sampling conditions. The percent of extractable organic compounds by dichloromethane for the ESP sample was higher than the filter sample and was dependent on collection temperature. The extracts were chemically fractionated into nine components according to a solvent partitioning scheme. These extracts and their fractions were used in *in vitro* biological studies.

INTRODUCTION

The potential health effects of diesel exhaust emissions should be determined with the expected increased usage of light duty diesel engines. In addition to chronic exposure studies with dilute diesel exhaust, the chemical and biological characteristics of diesel particles and the extracts would provide relevant information for the assessment of health hazards associated with inhaled diesel particles. Short term bioassays, such as the Ames test, have shown that diesel particulate extracts are mutagenic with varying degrees of biological activity dependent on engine types and fuels. Most of the biologically active compounds found on diesel particles are suspected to be hydrocarbon compounds formed either during the combustion process or adsorbed and condensed on the particles during the collection process. Experimental artifacts may exist if the diesel particles are not collected under proper conditions. The standard sampling method using appropriate filter papers may not be satisfactory for diesel particle collection if the biological activity of the particles is to be studied. The high pressure drop across the filter can lead to losses of volatile organic compounds on the particles on the filter due to the partial vacuum. Another serious problem associated with filter sampling may be the chemical conversion of the organic compounds on the particles by engine exhaust gases, such as nitrogen oxides and other reactive hydrocarbon compounds. Pitts, et al [1] reported that filters spiked with benzo[α]pyrene were found to be partially converted to nitrosubstituted compounds by 1 ppm nitrogen dioxide in laboratory studies. These factors may play important roles in the relative biological potency of diesel particles and their extracts.

In order to avoid some of these artifacts associated with filter sampling, electrostatic precipitation (ESP) was used in parallel to examine the role of collection method on the chemical and biological properties of diesel exhaust particles. The collection efficiency of ESP is almost as high as filtration, although the mechanisms of collection are distinctly different. Electrostatic collection of particles offers low pressure drop across the collector and allows the exhaust gases to pass over the collected particles instead of passing through them. Thus, losses of volatile compounds due to the partial vacuum and the mass transfer of gaseous hydrocarbons and nitrogen oxides to the particles should be reduced since only a small fraction of the gases is in contact with the diesel particles.

The objective of this study is to determine the effect of particle collection method on the chemical composition and biological activity of diesel particulate extracts. In this study, a 5.7L GM diesel engine was operated at 65 km/hr road load conditions. Type 2D federal compliance diesel fuel was used and a regular oil change was performed every 3,000 miles with 30W lubricating oil. Diesel particles collected by either ESP or filtration were extracted in a Soxhlet apparatus with dichloromethane. The diesel particulate extracts were chemically fractionated into nine components according to a solvent partitioning scheme. The biological activity associated with the extracts and their various fractions was studied using the Ames test.

EXPERIMENTAL METHODS

Collection of Diesel Particles

The sampling system for diesel particulate collection is shown in Fig. 1. A 5.7L GM diesel engine produced by Oldsmobile was controlled by a water brake dynamometer at 1350 rev/min and 96 N•m, representing 65 km/h road load cruise conditions. The exhaust from the engine passed through a normal passenger car exhaust system. A gate valve downstream of the muffler controlled the total diesel exhaust airflow and the collection temperature in the electrostatic precipitator. Parallel filter samples were obtained from the filter sampling ports using 47 mm Pallflex filter paper.

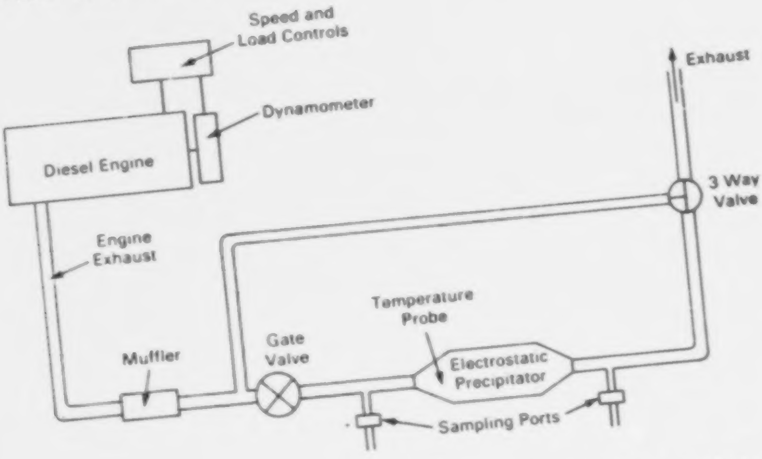

Figure 1 Electrostatic and filter collection of undiluted diesel particles from the exhaust of a 5.7 L GM diesel engine produced by Oldsmobile.

The basic principle of ESP collection of particles is a two-stage process: particle charging and particle collection. Particles entering the precipitator are charged electrically in a unipolar ion field provided by the corona discharge in the charging section. The charged particles are then immediately subjected to a high electric potential gradient and are driven towards the collection plates. A modified electrostatic precipitator (Trion, Inc., Model #424635) was used for the diesel particulate collection. The inlet and outlet of the precipitator were replaced by stainless steel reducers which provided a more developed flow field in the precipitator. Additional modifications included the isolation of the power supply module from the ESP mainframe (to prevent thermal degradation of the electrical and insulating components) and the installation of a removable front panel to gain access to the collector section. The collection temperature was usually maintained at normal tailpipe temperatures of 90-100°C. Direct readout of temperature was provided by a thermocouple placed in the ionization section of the precipitator. Particles deposited on the first 18 cm of the collection plates were shown to be within ±5°C of this temperature. Undiluted diesel exhaust particles were collected for fifteen minutes and the airflow and power to the ESP were turned off. The collection section was removed from the ESP and placed in a hood immediately. Particles collected on the first 18 cm of the collection plates were scraped off, placed in amber glass vials, sealed, and stored in a -80°C freezer. The collection and storage of particles were performed under these defined conditions to reduce the probability of losses and conversions of the biologically active compounds in the diesel particles.

Extraction of Diesel Particles

The organic compounds in the diesel exhaust particulates were extracted by dichloromethane (DCM) in a Soxhlet apparatus. The frozen samples collected either by ESP or filtration were allowed to reach room temperature by placing them in a dark hood for at least an hour. The cellulose extraction thimbles were also stabilized at 20°C and 45% relative humidity prior to weighing. Filter samples were placed vertically and ESP samples loaded directly in the thimbles. If necessary, a small pyrex rod was inserted into the extraction chamber to reduce the net volume and to increase the reflux rate to about 3-5 minutes/cycle. Routine extractions were performed at 40°C for four hours. When extraction was complete, the thimble was dried and restabilized at 20°C and 45% relative humidity to determine the mass lost. The total volume of the solution in the flask was reduced to about 5 mL by gentle evaporation with a mild jet of nitrogen. Additional rinses of the flask with 2 mL of redistilled dichloromethane were performed and the solution added to a tared vial. Finally, the solution was evaporated to dryness under nitrogen and the weight of extractable fraction determined.

Chemical Fractionation of Diesel Particulate Extracts

The diesel particulate extracts were fractionated into nine components according to a solvent partitioning scheme outlined in Fig. 2. The procedures used were based on the systematic extraction scheme of Novotny et al [2] in studies related to the identification of polynuclear aromatic compounds. The nine fractions obtained from the solvent partitioning scheme have been named WATER SOLUBLE, STRONG ACID, WEAK ACID, ACID SALT, BASE, BASE SALT, NEUTRAL-POLAR, NEUTRAL-NONPOLAR I and NEUTRAL-NONPOLAR II fractions. The WATER SOLUBLE fraction was obtained by extracting the diesel particulate extract in DCM with water. Soluble compounds such as acids, alcohols, ketones, aldehydes with low molecular weight, and inorganic salts should be present in this fraction. The organic layer was extracted with 5% $NaHCO_3$ solution. The aqueous $NaHCO_3$ layer was acidified, back extracted into dichloromethane, and dried in an evacuated rotary evaporator to yield a STRONG ACID fraction. Free organic acids such as sulfonic acids ($-SO_3H$), carboxylic acids ($-COOH$), and sulfinic acids ($-SO_2H$) belong to this category. The organic CH_2Cl_2 layer was treated with a strong base (NaOH) to yield a WEAK ACID fraction. Compounds such as

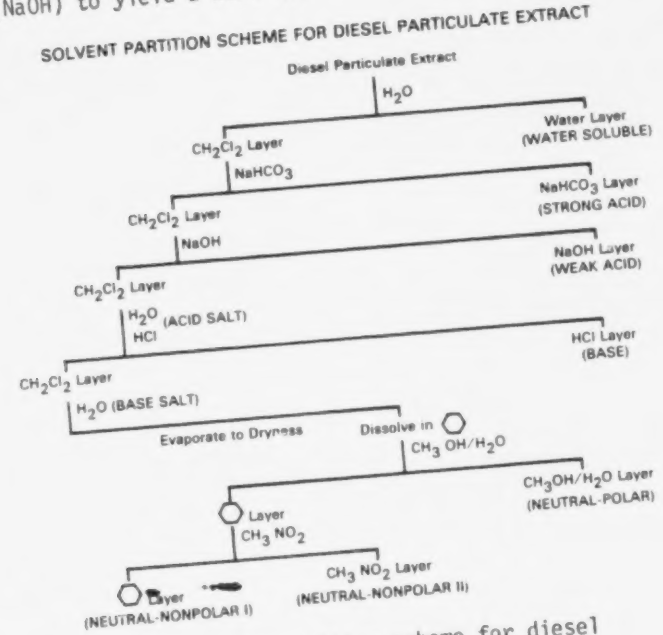
SOLVENT PARTITION SCHEME FOR DIESEL PARTICULATE EXTRACT
Diesel Particulate Extract
H2O
CH2Cl2 Layer
Water Layer (WATER SOLUBLE)
NaHCO3
CH2Cl2 Layer
NaHCO3 Layer (STRONG ACID)
NaOH
CH2Cl2 Layer
NaOH Layer (WEAK ACID)
H2O (ACID SALT)
HCl
CH2Cl2 Layer
HCl Layer (BASE)
H2O (BASE SALT)
Evaporate to Dryness
Dissolve in ⬡
CH3 OH/H2O
⬡ Layer
CH3OH/H2O Layer (NEUTRAL-POLAR)
CH3 NO2
⬡ Layer (NEUTRAL-NONPOLAR I)
CH3 NO2 Layer (NEUTRAL-NONPOLAR II)

Figure 2 Solvent partition scheme for diesel particulate extracts.

phenols (ArOH), primary and secondary nitro compounds, arylsulfonyl derivatives of primary amines ($ArSO_2NHR$), unsubstituted arylsulfonamides ($ArSO_2NH_2$), oximes ($-\overset{NOH}{\overset{\|}{C}}-$), thiophenol (ArSH), hydroxanic acid ($-\overset{O}{\overset{\|}{C}}-NHOH$) and active methylene compounds ($-\overset{O}{\overset{\|}{C}}-CH_2-\overset{O}{\overset{\|}{C}}-$) are typical examples in this group. The ACID SALT fraction was obtained next after the removal of both the STRONG and WEAK ACID fractions by treating with water. This fraction should consist of similar compounds as in the WEAK ACID fraction but with higher molecular weights. Addition of HCl to the organic layer produced the BASE fraction containing mainly amines and N-heterocyclic compounds. The organic CH_2Cl_2 layer was extracted with water to yield an aqueous layer named the BASE SALT fraction.

The remaining neutral compounds in the CH_2Cl_2 layer were evaporated to dryness and dissolved in cyclohexane. The solution was then extracted successively with solvents of decreasing polarity to yield three neutral fractions named NEUTRAL-POLAR, NEUTRAL-NONPOLAR I and NEUTRAL-NONPOLAR II compounds. Extraction with a 4:1 mixture of methanol/water yielded the NEUTRAL-POLAR fraction consisting of oxy-compounds such as aldehydes, ketones, alcohols, epoxides, nitrosamines, etc. Subsequent extraction with nitromethane gave the final two fractions. The cyclohexane layer provided the NEUTRAL-NONPOLAR I fraction with aliphatic and one-ring aromatic compounds. Finally, the nitromethane layer provided the NEUTRAL-NONPOLAR II fraction which should contain substituted and unsubstituted polynuclear aromatic hydrocarbons as well as aromatic nitro compounds.

Biological Activity of the Extracts

The biological activities of diesel particulate extracts and fractions were studied using a short term bacterial mutagenicity test system generally known as the Ames test. Experimental details of the assay are described in a separate report [5]. Since tester strain TA 98 was proven to be sensitive and stable in earlier experiments with diesel particulate extract, it was used to evaluate the biological activity of the extracts and fractions obtained from samples collected by ESP and filter samples. The mutagenic potency of the individual extract fractions would provide useful information regarding the significance of sampling methodology. Typically, the mutagenic activity recovered from all the fractions represented between 75-90% of the original extract.

RESULTS AND DISCUSSIONS

Collection of Diesel Particles

We have shown in pilot studies that the collection efficiency of diesel particles was 92% using an electrostatic sampler. In this study, a larger electrostatic precipitator was operated at about 300 L/min and 100°C. Under these sampling conditions, particle reentrainment and charging effects reduced the collection efficiency to 85%. It was possible to determine the collection efficiency only during the initial sampling period since the section of the exhaust line between the ESP and the downstream sampling port was never absolutely free of particles. Some of these particles can be reentrained to cause a decrease in the measured collection efficiency.

The most serious problem associated with the filter sampling of diesel particles was the potential chemical conversion of organic compounds to nitro-substituted compounds by the nitrogen oxides in the exhaust gases. Although electrostatic precipitation can effectively reduce these reactions with the exhaust gases passing over the collected particles, corona discharge and ozone formation during the particle charging process could alter the chemical species on the particles. In order to examine the role of ozone, a chemiluminescence ozone analyzer (Monitor Labs, Model #8410) was used to detect ozone at the outlet of the precipitator. When clean air was drawn through the ESP, 0.2 ppm of ozone was measured. However, no ozone was detected at a 5 ppb detection limit in the presence of undiluted diesel exhaust. Apparently, the 10% available oxygen in the diesel exhaust, the absorption of ozone by carbonaceous diesel particles, and the rapid reactions with reactive nitrogen oxides in the exhaust caused the dramatic reduction of ozone concentration. These considerations illustrated that advantages and limitations are associated with each sampling method. Thus, a thorough understanding of every particle collection mechanism and its potential artifacts is necessary in the interpretation of experimental data.

Extraction of Diesel Particles

Parallel samples obtained from both ESP and filter sampling at specific collection temperatures were extracted by dichloromethane. Results showing the percent of extractable compounds by weight to the collection temperature were given in Fig. 3. An increase in the extractables was found for the ESP sample compared to the filter sample at

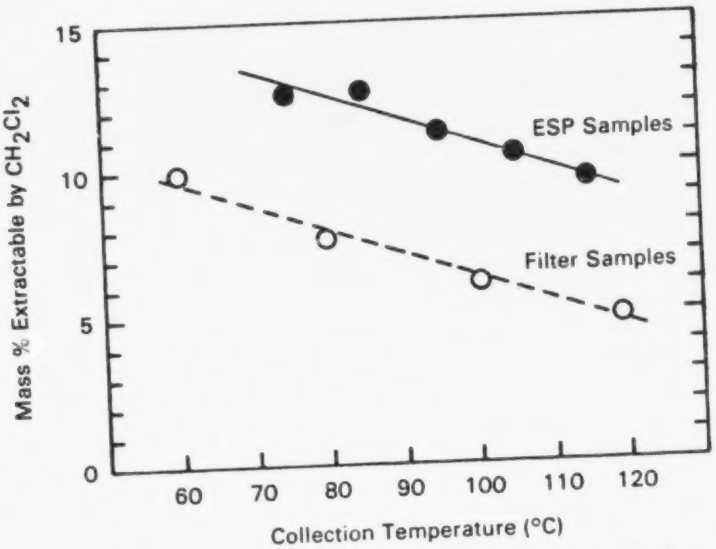

Figure 3 Percent of extractable compounds in diesel particles collected by ESP or filtration. Soxhlet extraction for four hours at 40°C was performed with dichloromethane as the solvent.

the same collection temperature. Both diesel particulate extracts exhibited similar dependence on collection temperature, i.e., an increase in % extractables at lower temperatures. This was reasonable since various gaseous hydrocarbon compounds could easily be adsorbed or condensed onto the diesel particles.

The characteristics of undiluted diesel particles at tailpipe conditions were studied since it would allow us to examine the effects of dilution and cooling of diesel exhaust. At the normal exhaust temperatures of 100°C at the tailpipe, 11% was extracted from the ESP sample but only 6.2% was obtained in the filter sample. Since both samples were collected simultaneously, the differences observed must be related to differences in the collection methodology. Volatile compounds which could be lost in the filters would be retained in the ESP. Direct acting mutagenic compounds may be formed by chemical conversion by the exhaust gases in the filter. Moreover, formation of new compounds during the particle charging process may be possible in the presence of excess ions provided by the corona discharge. Detailed chemical fractionation of the diesel particulate extracts were therefore necessary to determine differences in the chemical profiles due to the collection method.

Chemical Profiles of the Particulate Extracts

Parallel samples collected simultaneously by ESP and filtration at 90-100°C were fractionated according to the solvent partitioning scheme described earlier. Only paired samples were compared to avoid variations due to other experimental parameters. Results indicated that more than 60% of the extractables were found in the NEUTRAL-NONPOLAR I fraction (Fig. 4). This fraction consisted primarily of aliphatic and one-ring aromatic compounds. Initial data indicated that the overall chemical profiles were quite similar with the exception of the ACID SALT fraction. Significantly greater amounts were found in this fraction which accounted for most of the increase in extractables in the ESP sample. Since most of the compounds in this ACID SALT fraction were not expected to be volatile, the increase must be attributed to some characteristics associated with electrostatic collection. The corona discharge could provide an abundance of ions in the charging section of the electrostatic precipitator. Chemical kinetics studies have shown that free radicals are formed under field ionization [3,4]. These free radicals can undergo rapid chemical reactions to form larger molecules. Therefore, it was conceivable to see some conversion

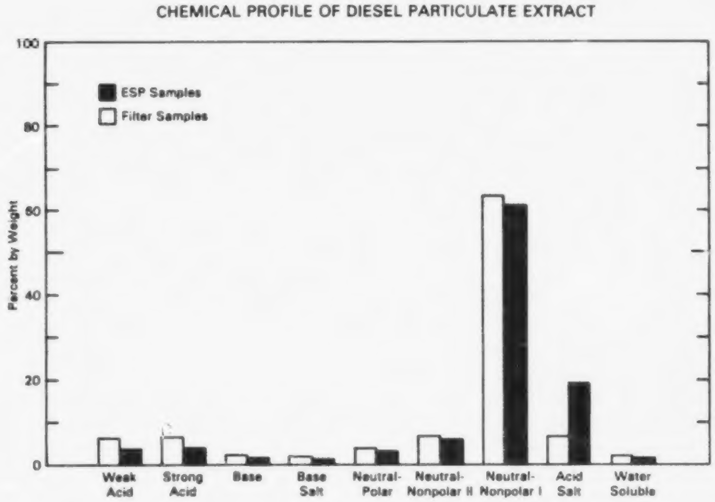
CHEMICAL PROFILE OF DIESEL PARTICULATE EXTRACT

Figure 4 Chemical fractions of diesel particulate extracts for both ESP and filter samples. The NEUTRAL-NONPOLAR I fraction accounted for more than 60% of the extractables.

of the WEAK ACID compounds to similar compounds with higher molecular weights in the ACID SALT fractions in the ESP sample. This mechanism would also lead to the decrease in the amount of WEAK ACID and STRONG ACID fractions for the ESP samples. Additional paired samples collected under the best reproducible conditions are being evaluated to determine the variations in the data. With regard to the formation of nitro-substituted compounds on the filter samples due to the nitrogen oxides, there was very little difference in the amount of extract fraction. However, the specific bioactivity of these extract fractions may be different and must be examined in *in vitro* test systems such as the Ames test.

In Vitro Biological Activity of the Extract Fractions

Although the ESP sample extract exhibited a significant increase in the amount of extractables in the ACID SALT fraction, no activity was detected in the Ames test. By comparison, the same fraction from the filter sample extract accounted for 1.5% of the total mutagenic activity. Apparently, charging effects during the electrostatic collection process enabled the conversion of some of the compounds in the WEAK ACID and STRONG ACID fractions to non-mutagenic compounds classified under the ACID SALT fraction. Thus, increased amounts of extractable mass in the ESP sample do not give rise to a corresponding increase in the bioactivity in the diesel particulate extract. The overall biological activity of the extracts for ESP and filter samples (expressed as net revertants/mg of particles basis) was quite similar [5].

The subtle effects of the sampling methodology on the bioactivity of the extract can be seen by examining the specific activity of the most active extract fractions (Fig. 5). Higher specific activities were observed in the filter samples in the WEAK and STRONG acid fractions. Significant increases in specific activity were seen in the filter sample extracts tested under the inactivated system for both NEUTRAL-POLAR and NEUTRAL NON-POLAR II fractions. This strongly suggested the existence of more direct acting mutagens in the filter sample. Work is still in progress to identify the active compounds in these fractions.

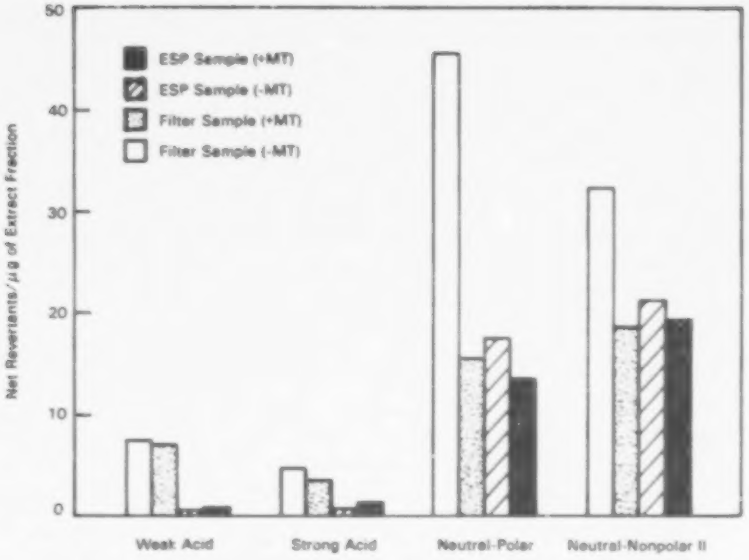

Figure 5 Specific activity of diesel particulate extract fractions determined by the Ames test. +MT indicates metabolic activation with S9, -MT indicates no activation.

SUMMARY

1. The amount of extractable compounds from the diesel exhaust particles depended on collection temperature and collection method. Under the same collection temperature, more extractables were found in diesel particles collected by electrostatic precipitation (ESP) than filtration.

2. The chemical profiles of the two particulate extracts were similar with over 60% of the extractable mass classified as a NEUTRAL-NONPOLAR I fraction. This fraction was obtained from a solvent partitioning scheme and should contain aliphatic and one-ring aromatic compounds.

3. Most of the mass increase in the extractables in the ESP sample was found in an ACID SALT fraction and could be attributed to charging effects. Although there was more than a two-fold increase in this fraction, no bioactivity was observed in the Ames test.

4. Bioactive compounds determined by the Ames test were found in the NEUTRAL-POLAR and NEUTRAL-NONPOLAR II fractions. These fractions accounted for more than 70% and 90% of the bioactivity in the filter and ESP samples, respectively. However, only 10-15% by weight of the extractables belonged to these fractions.

CONCLUSIONS

We have examined the effect of sampling methodology on the chemical composition and biological activity associated with the diesel particulate extracts. Electrostatic precipitation gave a larger amount of extractables with most of the increase in an ACID SALT fraction. This fraction was subsequently shown to be inactive in the Ames test. Filter sample extracts do exhibit higher specific activities in some fractions and may be attributed to the presence of some direct acting mutagens formed by reaction with the exhaust gases. On the other hand, charging effects during electrostatic precipitation may convert some of the mutagenic compounds to less mutagenic forms. Additional experiments are required to determine the dominant mechanism. However, the overall biological activity of the extracts (expressed as net revertants/mg of particles basis) was quite similar. These subtle differences due to the sampling methodology must be recognized in the assessment of health hazards associated with inhaled particles. Due to the presence of hydrocarbon compounds and nitrogen oxides in the gaseous phase in urban atmospheres [6], routine filter samples drawn for extended periods of time could create some direct acting mutagens in the extracts. Therefore, studies involving short term bioassays of particulate extracts [7,8] would provide relevant but only relative information concerning the biological hazards of airborne particles.

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General Discussion

W. BALGORD: You mentioned several types of possible artifacts with filters; I wonder if you had considered the possibility of your electrostatic precipitator producing ozone. Did you measure that?

T. CHAN: Yes, first of all we did some ozone measurements before we installed the precipitator in the collection system. In the presence of clean air we can measure up to 0.2 PPM of ozone. However, in the presence of undiluted diesel exhaust, the amount of ozone was below our detection limit which is 0.2 PPB. Apparently what happened is the ozone is taken out by the hydrogen dioxide in the exhaust gas or could be absorbed by the particles. In this case, considering that the concentration of the nitrogen oxide in the exhaust is so high, the 0.2 PPM of ozone would not be a problem.

A. KOLBER: Can you give us an estimate of what percentage the polar neutral fraction represents of your total extractable mass?

T. CHAN: It was 60 percent.

A. KOLBER: It represented 60 percent of the polar?

T. CHAN: Sixty percent of the extractable mass. I indicate here also that the percent, by mass, of the active fractions only amounts to 10 - 15 percent of the extractable mass for diesel particles.

R. DALLEY: Could you tell us why you looked at the raw exhaust and also what type of filter was used?

T. CHAN: I will answer the second question first. We used pallflex filters, and we chose to sample from the undiluted exhaust simply because we wanted it to determine the activity and the chemical composition at the point of emission.

R. DALLEY: How is the temperature controlled?

T. CHAN: In the case of a precipitator, we could control it with the gate valve upstream of the precipitator, which controls the flow rate into the precipitator. In case of a filter, we controlled it with a gate valve behind the filter.

D. KITTELSON: Did you use a positive or a negative corrider with the percipitator, and did you try changing the corrider polarity? That would change the sources of compounds that you might be forming in the corrider and thus perhaps give rise to artifact formation.

T. CHAN: We used a positive corrider and since the percipitator was available from this company, we have no way of switching polarity. We would like to, but we have no way of doing that. We also considered the possibility of not using a target section, but we are in the process of working out the details of that.

J. HUISINGH: We have done some experiments over the last year with an electrostatic precipitator in ambient air and have compared charging the corrider two different ways. We have measured ozone and have found no difference in mutagenic activity in three different locations, including on top of a coke oven. The chemistry of the PAH's were also characterized and we essentially found no major differences, so it would appear that, if similar to the diesel exhaust, ozone would not be a problem with diesel. I have a question about your fractions. You saw a lot more activity than we have seen in the nonpolar neutral fractions, and yet it was direct acting activity. When we tried a fractionation scheme to this, we saw the same thing and assumed that we got spill-over of the oxygenated type materials into the PAH fraction. Do you think that it was the PAH's that we were seeing?

T. CHAN: I don't think so.

J. HUISINGH: The PAH's would be indirect acting and your fractions were direct acting.

T. CHAN: Dr. Siak will give the next paper to discuss details of the biological testing. I think it would be appropriate for him to answer that question after his talk.

R. BRADOW: We have conducted a similar experiment. This morning I described a split flow system wherein the diluted raw exhaust was compared to a filtered exhaust with a filter held at 100 degrees centigrade. We obtained an electrostatic precipitator very similar to the one used by General Motors and have reconducted that same experiment electrostatic precipitator plate (ESP). Generally speaking, we got about the same mutagenicity from the Pallflex filter that we got in those that were obtained from extracts of the (ESP) plates.

J. HUISINGH: Is that after diluting?

R. BRADOW: No, that was raw exhaust. So our conclusion was that the diluted sample was very similar, slightly lower in activity, because at the somewhat lower temperature there was larger retention of polyaromatic hydrocarbon so the material was less diluted. Pallflex filter samples and ESP samples collected at that location were essentially comparable by HPLC analyses as well as by the Ames Test. So in the raw exhaust, we found no difference between ESP and the Pallflex filter.

DIESEL PARTICULATE EXTRACTS IN BACTERIAL TEST SYSTEMS

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ABSTRACT

The Ames bacterial mutagenicity test system was used to evaluate parameters which may affect the mutagenic activity of diesel particulate extracts. The optimal extraction conditions, extractability of mutagens by simulated biological fluids and the effect of collection method were investigated. The role of solvent was examined by extracting diesel particles with methanol, acetone, cyclohexane, ethyl acetate, n-hexane, dichloromethane, benzene and a benzene-ethanol mixture. Of these, the dichloromethane extract exhibited the highest activity in the Ames test, although methanol yielded the largest extractable mass. Diesel particles were also extracted by dimethyl sulfoxide (DMSO) and four other simulated biological fluids for 48 hours at 25°, 37° and 45°C to study the effects of temperature. The mutagenic activity of the DMSO extract began to decline at temperatures higher than 37°C after 8 hours of incubation. Fetal calf serum was the only simulated biological fluid which eluted mutagenic activity from the particles. No activity was detected in the 0.5% bovine serum albumin, simulated lung surfactant and saline extracts. Diesel particles collected by electrostatic precipitation (ESP) and filtration were studied. The mutagenic activities of both extracts were comparable when expressed as revertants per mg of particle. After the extracts were separated into nine fractions by a solvent partitioning scheme, the majority of the activity was found in the neutral-nonpolar II, neutral polar, strong acid and weak acid fractions. The acid salt fraction from the ESP sample was inactive. These results demonstrate that differences in the extraction conditions can result in differences in the mutagenic activity of diesel particulate extract. Since the mutagens in the extracts are not readily extractable by simulated biological fluids, the question of bioavailability of mutagens in diesel particles must be considered in the final assessment of their potential effects in biological systems and organisms.

INTRODUCTION

Recent studies have indicated that the organic solvent extracts of diesel particles exhibited different mutagenic response in the Ames mutagenicity test. The variability in the findings may be attributed, in part, to differences in the composition of the extracted compounds. Although Huisingh et al [1] found that after extraction of diesel particles with dichloromethane, the extract was mutagenic in the *Salmonella typhimurium* mutagenicity test [2], McGrath et al [3] observed that diesel particles were only slightly mutagenic when the particles were tested as a dimethyl sulfoxide suspension. These observations suggest that the mutagens in diesel particles have different extractability in different solvents. Similar findings were observed in fly ash studies [4]. Also, it has been shown that serum extracts only small quantities of benzo[a]pyrene from benzo[a]pyrene-enriched carbon black particles [5]. Moreover, the results obtained in the studies with organic solvents do not reflect the conditions *in vivo*. This study was designed to examine the roles of various parameters on the Ames mutagenicity test. The optimal extraction conditions, the extractability of the mutagens in simulated biological fluids, and the effect of particulate collection method were investigated.

EXPERIMENTAL METHODS

Collection of Diesel Particles

The diesel particles were collected either by a Pallflex filter or by an electrostatic precipitator [6] from a GM 5.7 L diesel engine. The engine was operated on a water brake dynamometer at 1350 rev/min and 96 N·m, representing 65 km/h road-load cruise conditions. The particles were collected from the undiluted exhaust at the normal tail-pipe temperature of 100°±5°C. Type 2D federal compliance diesel fuel was used in this study.

Preparation of Simulated Biological Fluids

Four solutions were prepared to simulate body fluids. Saline (SLN) was prepared as 0.9% sodium chloride in deionized water. A 0.5% bovine serum albumin (BSA) in saline was used to simulate the proteinaceous material in body fluids. To simulate the surfactant layer of the alveoli, dipalmitoyl-2-lecithin, palmitic acid, stearic acid and tripalmitin, and cholesterol were used to generate simulated lung surfactant (SLS) vesicles according to the method of Schroit and Pagano [7]. Fetal calf serum (FCS) was chosen to represent body fluids with complex properties.

Extraction Procedures

Diesel particles (200-300 mg) were extracted with 150 mL dichloromethane (DCM) or other solvents (Table 1) in a Soxhlet apparatus for 4 hours accounting for 20-25 solvent-wash cycles. The volume was reduced to 10-15 mL under vacuum in a Rotavapor (Buchi) and the remaining solvent was evaporated under nitrogen to dryness to determine the extractable mass. The extracts were stored at -80°C and were subsequently redissolved in DMSO for use in mutagenicity tests.

The concentration of diesel particles used in the SLN, 0.5% BSA, FCS, SLS and dimethyl sulfoxide (DMSO), was 5 mg/mL. The extractions were performed in a shaking water bath at the desired temperature (25°C, 37°C, or 45°C). Two milliliters of the extraction mixture were removed at selected time intervals and centrifuged at 10 000 g and room temperature for 30 minutes. The samples were frozen at -80°C until they were examined in the mutagenicity test.

Preliminary experiments indicated that bacterial contamination may occur in FCS extract after prolonged incubation. This problem was eliminated either by incubating the extraction mixtures at 45°C or by adding ampicillin (50 μg/mL) to the extraction mixture. Ampicillin had no effect on the mutagenicity of the extract as demonstrated by the negative controls used in each test.

For the multiple solvent-wash cycle study, 2 grams of diesel particles were extracted with 50 mL of dichloromethane for 10 minutes under constant agitation at room temperature. After the extract was separated from the particles by centrifugation, the remaining particles were re-extracted with the same volume of fresh solvent as described above. This procedure was repeated ten times. The extracts were then evaporated to dryness and stored at -80°C as described earlier.

Bacterial Mutagenicity Test

The bacterial mutagenicity test used in this investigation was essentially the same as described by Ames et al [2]. Preliminary experiments indicated that extracts of diesel particles elicited significant mutagenic activity in the TA1538, TA98 and TA100 strains of *Salmonella typhimurium*. Of these, TA1538 and TA98 gave the most reproducible responses. In addition, TA98 is resistant to ampicillin whereas the TA1538 strain is not. Consequently, TA98 strain was used in this study since ampicillin had to be used in conjunction with the simulated biological fluids.

Bacteria were grown in nutrient broth (TN) containing 0.9% sodium chloride, 0.4% tryptone, 0.25% yeast extract, and 0.1% glucose. The cultures were kept at 37°C in a shaker water bath and grown to an optical density (A_{425}) of 1.5 to 2.5. The bacteria were harvested by centrifugation at 10 000 g and 25°C. The cell pellets were resuspended in sterile saline supplemented with 10% TN broth to an optical density of 2, which represented a cell density of 2-5 x 10^8/mL. The suspensions were kept on ice during the experiment to maintain the viability of the bacteria.

The Aroclor 1254-induced rat liver enzyme preparation, S9, was purchased from Litton Bionetics, Kensington, Maryland. A reaction mixture was prepared according to Ames et al [2] and sterilized by filtration through a Millipore® filter (0.45 μm pore size). The final volume applied was 20 μL S9 in 0.5 mL of reaction mixture per plate.

The test compound was first introduced in a tube containing 2 mL molten agar overlay (0.6% agar, 0.05 mM histidine, 0.05 mM biotin, and 0.9% NaCl). Then, 0.1 mL of the bacterial suspension was added and mixed thoroughly. If the test required metabolic transformation, 0.5 mL of the S9 mixture was added before mixing. This mixture was poured onto Vogel-Bonner E medium and allowed to solidify. The plates were incubated at 37°C for 48 hours, and the number of colonies was determined by an automated counter (Biotran Colony Counter).

Five doses of each extract were used to establish the dose-response curve, and the activity of each dose was determined by duplicate plates. The mutagenic activity of the extracts was expressed as: 1) extract activity (his$^+$ revertant per mg extract for DCM extract or his$^+$ revertant per mL extract solution for simulated biological fluids and DMSO extracts), and 2) specific activity (his$^+$ revertant per mg particle).

The extract activity was calculated from the slope of the linear portion of the dose-response curve: $y = a + bx$.

1. For DCM extracts:

   y is the number of revertants per plate,
   x is the concentration of the extract per plate, (mg extract/plate)
   a is the intercept and b is the slope of the regression line (revertants/mg extract).

2. For simulated biological fluids and DMSO extracts:

   y is the number of revertants per plate,
   x is the volume of extract solution per plate (mL/plate)
   a is the intercept and b is the slope of the regression line (revertants/mL extract solution).

The specific activity (S.A.) is calculated as follows:

1. For DCM extracts:

   S.A. (his$^+$ revertants/mg particle) =

   b(rev/mg) · Percentage Extractable Mass (%)

2. For simulated biological fluids and DMSO extracts:

$$\text{S.A.} = \frac{b\ (\text{rev/mL extract solution})}{c\ (\text{mg particle/mL extract solution})}$$

   where c is the mass of particles per volume of extract solution (mg particle/mL extract solution).

RESULTS

Optimal Extraction Conditions

The choice of extraction solvent, extraction time and extraction temperature were examined experimentally. A total of eight organic solvents ranging from methanol to a benzene-ethanol azeotrope were used. Table 1 lists the solvents used along with their respective dielectric constants and boiling points. First, the amount of extractable mass seemed to correlate with the polarity of the solvents. More polar solvents extracted more mass than the less polar solvents as shown in Figure 1. However, the extractable mass did not correlate with the mutagenic activity of the extracts. Methanol yielded the most

Table 1

Physical Characteristics of the Solvent

Solvent	Dielectric Constant (ε) at 25°C	Boiling Point (°C)
Water	78.54	100
Methanol	32.63	64.96
Acetone	20.7	56.2
Dichloromethane	8.895	40
Ethyl acetate	6.02	77.06
Benzene	2.274	80.1
Cyclohexane	2.015	80.74
n-Hexane	1.882	68.95

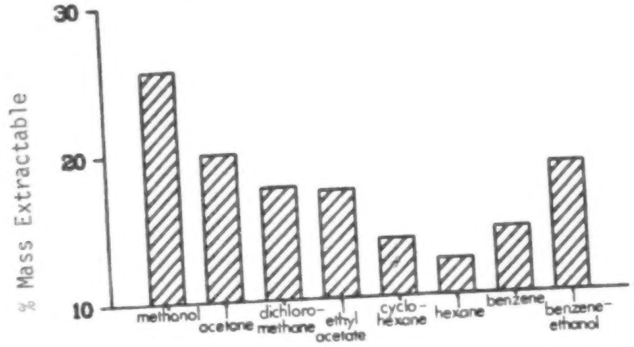

Figure 1 Percent of extractable mass of diesel particles by organic solvents.

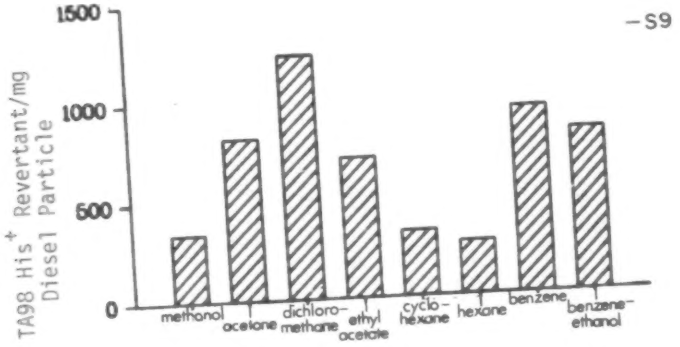

Figure 2 Mutagenic activity of diesel particulate extracts expressed as TA98 net revertants/mg of particle without S9 activation.

extractable mass from the diesel particles, but the extract was not the most active in the Ames test. Cyclohexane and n-hexane extracts were also relatively less active. Dichloromethane extract was found to be most active, followed by the benzene and the benzene-ethanol extracts. Thus, DCM seems to be the best solvent for the extraction of mutagenic activity from diesel particles, although it does not remove the maximum amount of extractable organic matter from the particles (Figure 2).

Secondly, the number of solvent wash cycles required to remove most of the mutagenic activity from the diesel particles was examined from the successive dichloromethane extracts obtained from the 10 minute washes. Figure 3 shows the cumulative percentage of the extractable mass and mutagenic activity of the extracts as a function of the number of successive washes. More than 90% of the extractable mass and mutagenic activity were recovered after only 4 washes. After 10 washes, the mass and activity extracted from the particles were within 1% of the amount removed by Soxhlet extraction over 20-25 solvent wash cycles. From these results, we established that a minimum of 10 solvent wash cycles was sufficient to elute most of the mutagenic activity from the diesel particles.

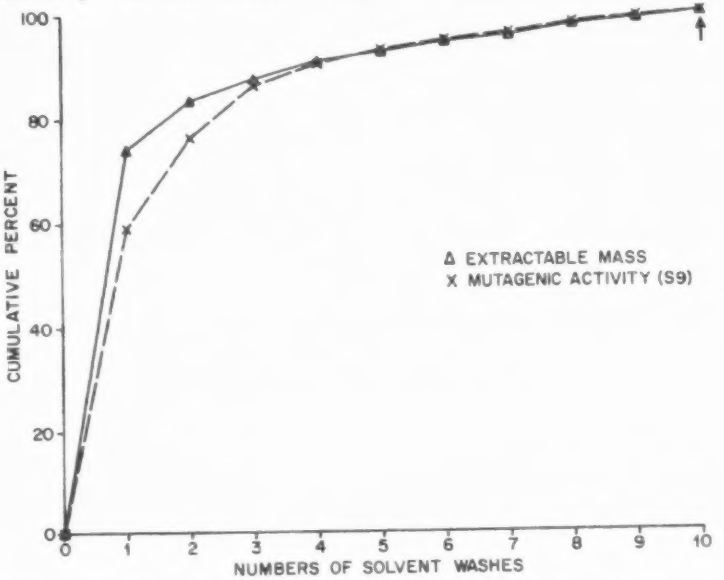

Figure 3 Cumulative percentage of extractable mass and mutagenic activity from diesel particle by multiple solvent wash cycles.

Thirdly, the effect of extraction temperature was investigated by incubating the diesel particles with dimethyl sulfoxide at 25°, 37° and 45°C. The mutagenic activities of these extracts were determined up to 48 hours. The activity of the extracts at these temperatures reached the maximal level after 1 to 2 hours of extraction (Figure 4). At 25°C, the activity remained stable throughout the extraction duration; whereas the activity of the extracts at 37° and 45°C began to decline after 6-8 hours of extraction. These results indicated that some of the mutagenic activity in the diesel particles was affected at the higher extraction temperatures, a moderately low 37-45°C. The combination of heat and oxidation effects may be responsible for the decline of activity in the extracts.

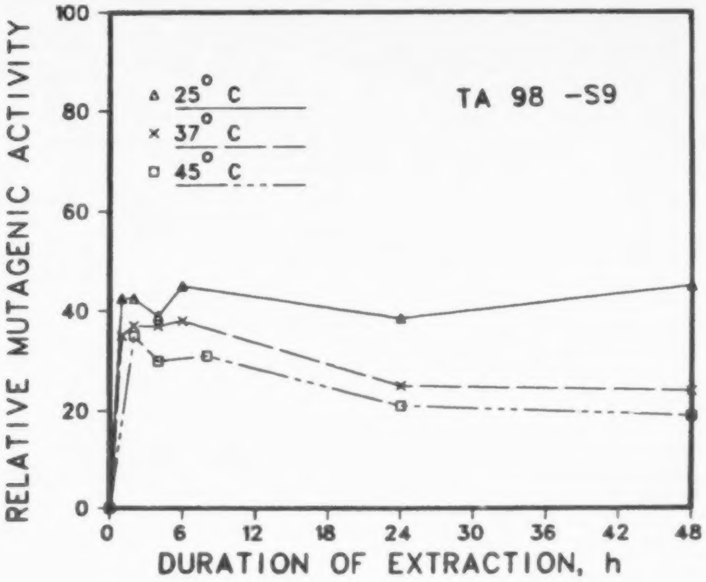

Figure 4 The effect of extraction temperatures on the mutagenic activity of the DMSO diesel particulate extracts. The activity of DCM extract was taken as 100%.

Extractability of Mutagens in Simulated Biological Fluids

In order to compare the activity of the extracts from different solvents, the DCM extract activity was used as a reference to calculate the relative mutagenic activity of these extracts. Figure 5 shows the relative mutagenic activity of the FCS and the DMSO extracts at different times during one extraction at 45°C. The DMSO extract reached its maximum level of activity after two hours of extraction, and the activity began to decline after 8 hours of exposure to 45°C. On the other hand, the activity of the FCS extract increased slowly during the entire period of extraction. On an average, 6 ± 5.2% of the activity was eluted by FCS after 48 hours at 45°C in five experiments and the range was 3.6 to 12.6%.

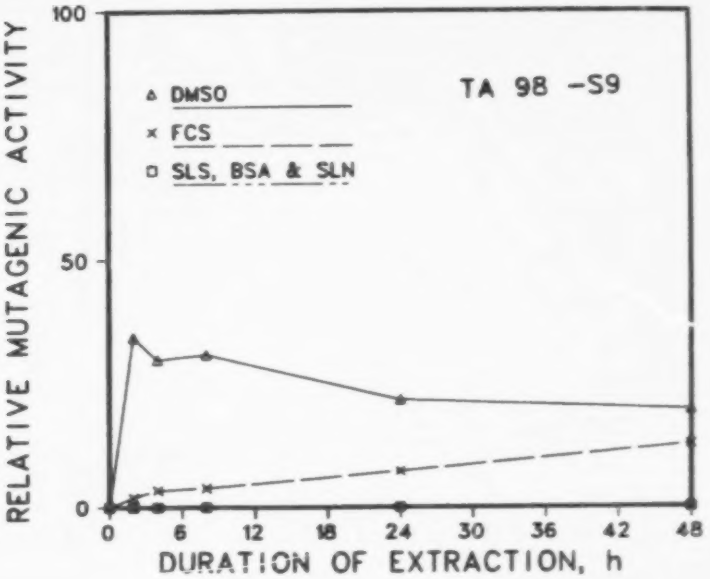

Figure 5 The relative mutagenic activity of diesel particle extracts at 45°C versus time. The activity of DCM extract was taken as 100%.

A typical dose-response curve of the mutagenic activity of the DMSO and FCS extracts is shown in Figure 6. Each point of the curve was determined from four plates. The durations of extraction were 2 hours for DMSO and 48 hours for FCS. Extracts obtained at these durations exhibited the highest activity. Note that the activity of the FCS extract was much lower than the DMSO extract, both with and without metabolic transformation despite the more than twenty times longer extraction period.

Five experiments were repeated with different batches of diesel particles, and the results are summarized in Figure 7. The data again indicated that dichloromethane is much more effective in extracting the mutagens from diesel particles than dimethylsulfoxide and that the simulated biological fluids remove little or no mutagens from diesel particles.

Comparison of Mutagenic Activity of Diesel Particles Collected by Electrostatic Precipitator and Filtration

The effect of collection method on the chemical composition and biological activity of diesel particles was described in a separate report [6]. Although the percentage of extractable mass from the electrostatically collected sample (ESP) was greater than that obtained from the filter sample, the mutagenic activities of the extracts expressed as net revertants/mg of diesel particles were comparable as shown in Table 2. Most of the increase in extractable mass for the ESP sample was found in the acid salt fraction obtained after chemical fractionation of the extract [6]. Mutagenic activities for all nine extract fractions were

Table 2

TA98 Specific Activity of Diesel Particles
Collected at 100°C by ESP and Filtration

Sample	his^+ net revertants/mg particle n*	-S9	+S9
Diesel Particle (ESP)	12	440±105**	419±175
Diesel Particle (Pallflex filter)	4	367± 45	307± 22

* number of experiments
**mean ± standard deviations
Positive Controls (n=6):
2-nitrofluorene (2.5 μg) (-S9) 368±22
2-aminoanthracene (2.5 μg) (+S9) 1047±91

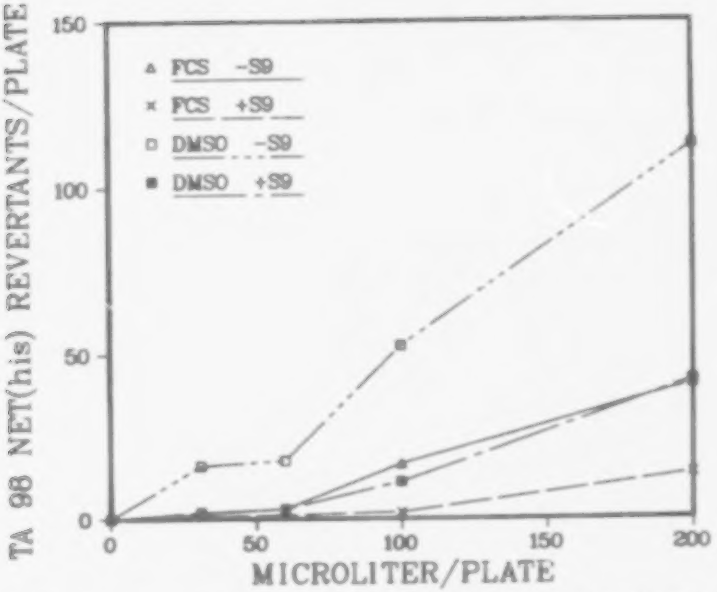

Figure 6 Dose-response curves of DMSO and FCS extracts of diesel particles.

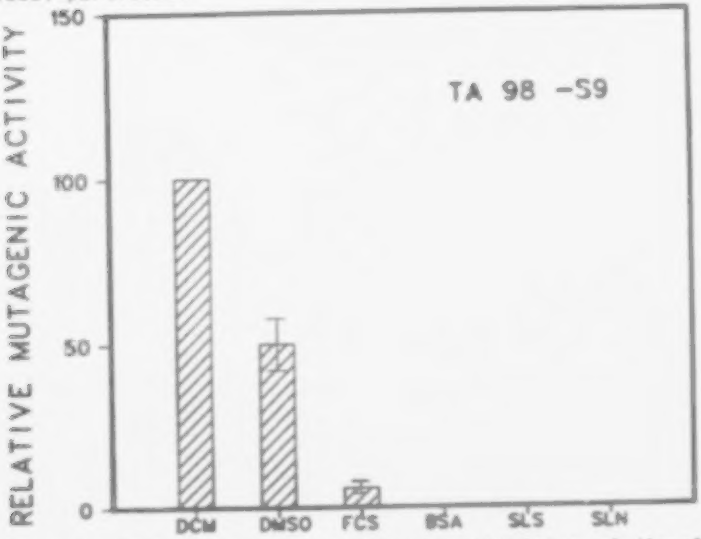

Figure 7 Comparison of the mutagenic activities of diesel particulate extracts DCM (dichloromethane), DMSO (dimethyl sulfoxide), FCS (fetal calf serum), BSA (0.5% bovine serum albumin), SLS (simulated lung surfactant), and SLN (saline).

determined in the Ames test where the acid salt fraction from the ESP sample was found to be inactive. More than 90% of the biological activity was accounted for in the neutral-nonpolar II, neutral polar, weak and strong acid fractions. The mutagenic activity of each extract fraction is presented in Figures 8 and 9 as a percent of the total activity of all the extract fractions.

DISCUSSION

Among the solvents used in this study, DCM was the most effective solvent to elute the mutagens from diesel particles. In contrast, DMSO showed only a moderate ability to extract mutagens from the particles. FCS extracted 6 ± 5.2% (range 3.6 to 12.6%) of the activity found in the DCM extracts, whereas no activity could be detected in the 0.5% BSA extracts. The difference in mutagenic activity between the FCS and the 0.5% BSA extracts may be attributed to the presence of lipoproteins and phospholipids in the fetal calf serum and to the fact that the lipids and lipoproteins in the FCS may extract mutagens from the particles which are not extractable by proteins alone. Indeed, benzo-[a]pyrene was found to associate with serum lipoproteins *in vitro* [8]. However, the fact that the simulated lung surfactant (SLS) lacks any mutagenic activity despite the presence of significant amounts of phospholipids does not fully support this explanation.

Although earlier studies have reported that diesel particulate extracts obtained by powerful organic solvents have a significant mutagenic activity, the results of this study indicate that the mutagenic activity in diesel particles is not readily removable by simulated biological fluids. In addition, 90-95% of the mutagenic activity of the diesel particulate extract was contained in only two fractions consisting of polynuclear aromatic compounds. Since the chemical species in these fractions are expected to be only slightly soluble in aqueous solutions, the question of bioavailability of the mutagens in diesel particles must be considered in the final assessment of any potential effects of diesel particles in biological systems and organisms.

The effect of the extraction temperature on the mutagenic activities of DMSO extracts is important in the preparation of diesel particulate extracts for chemical and biological analyses. Indeed, the mutagens extracted by DMSO at 45°C for more than 8 hours were shown to be unstable and may lose a significant amount of their activity after long exposures to this temperature. Therefore, caution should be exercised in the extraction of diesel particles for testing of mutagenic effects if losses of biological activity are to be minimized. On the other hand, the

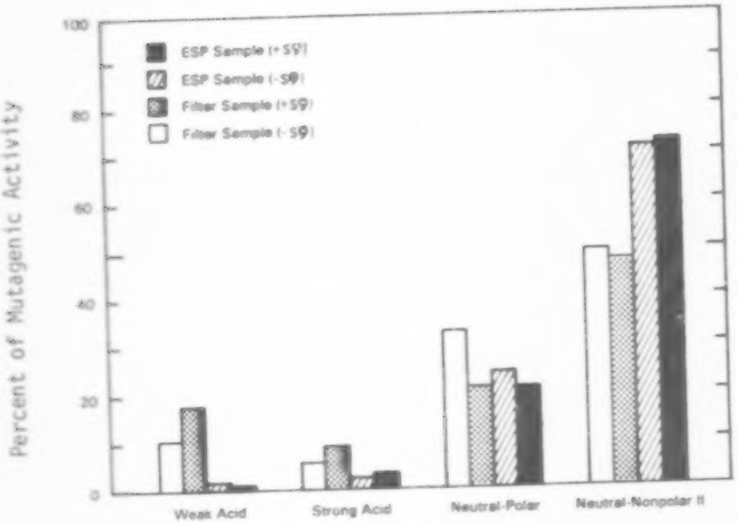

Figure 8 Most of the mutagenic activity of the diesel particulate extracts was found in four extract fractions. ESP indicated sample was collected by electrostatic precipitation. +S9 and -S9 indicated S9 activation or no activation in the Ames test using TA98.

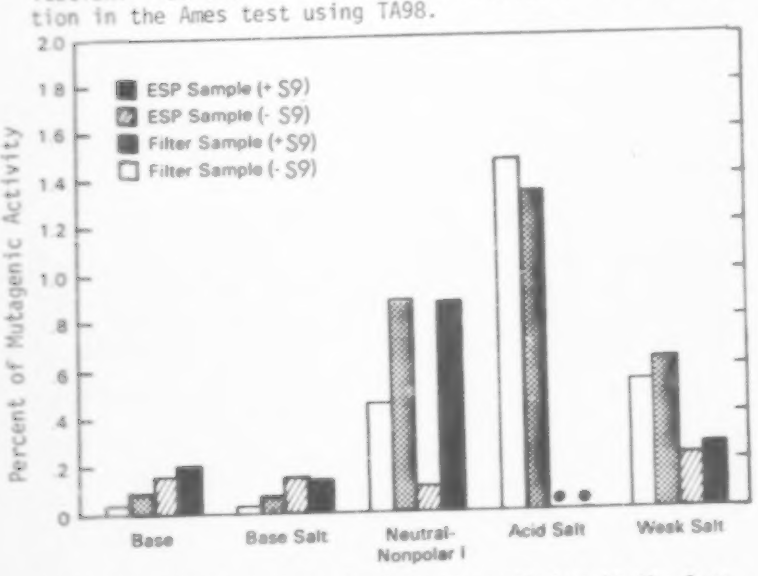

Figure 9 Contribution of mutagenic activity in the less active extract fractions of the diesel particulate extracts.
*Below detection limit

bioactivity expressed on a particle weight basis is not affected by the collection methods studied.

CONCLUSIONS

The *Salmonella typhimurium* mutagenicity test system is a useful technique to study the mutagenic properties of diesel particulate extracts. However, temperature and oxidation effects can alter the mutagenic activity of the extract. Caution should be exercised during the extraction and handling of the diesel particulate extracts in order to prevent any loss of biological activity. The choice of solvent in the extraction is also critical. Although methanol extracted the greatest amount of mass from the diesel particles, the dichloromethane extract was found to be most active in the Ames test. The acetone, cyclohexane and benzene extracts exhibited less activity compared to the DCM extract. The particulate collection method and the sampling conditions must also be examined and standardized to avoid sampling artifacts.

No mutagenic activity could be detected in the 0.5% bovine serum albumin, simulated lung surfactant, and saline extracts, even at a particulate concentration of 5-10 mg/mL in the extraction mixture. Only the fetal calf serum displayed the ability to extract some mutagenic activity from diesel particles. These results indicated that the mutagens in diesel particles would not be readily available *in vivo*. Thus, the question regarding the biological fate of inhaled diesel particles and the metabolic transformation of the active compounds found in the diesel particulate extracts should be addressed in future studies.

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General Discussion

R. BRADOW: Recently, one piece of evidence has come out of our work relative to the extraction of the diesel with protic-solvents. Apparently this happens both with methanol extraction are with extractions with benzene/methanol or toluene/methanol. The protic solvents seem to have a tendency to extract fairly well the sulphate and nitrate ions, inorganic anions. We avoided that approach fearing some toxicity in the biological testing due to the elevated levels of electrolytes in the medium. Have you experienced such effects? Our endochromatography indicates that a fairly substantial level of sulphate can be obtained.

J. SIAK: Most of the studies in our department have used BZMA X-rays. We have done some other experiments where we extracted particles with methanol first, coextracted it, and re-extracted it with dichloride methane. The dichloride methane extract by the secondary extraction has brought very high activity in a low percentage recovery. They are very active, and I think we have not looked into the problem. I think the methane cold wash will remove a lot of salt and then you can clean your system quite well by using that two-step procedure.

R. BRADOW: Yes, initially the attack that we made on that similar problem was to extract with DCM and then follow it subsequently with acetonitrile, but even a nonprotic, highly polar solvent such as acetonitrile was fairly effective in removing the inorganic anions. Ultimately we abandoned that procedure because of the problems in distinguishing between the organic material and the inorganic substance.

P. NETTESHEIM: What are the conclusions you drew from the fact that the particles seem to extract so poorly in simulated body fluids?

J. SIAK: You can look at our chemical analyses. Most of them are what we call neutral polar, neutral nonpolar - you don't realize they are not dissoluble in aqueous solutions, to the best of my interpretation.

J. HUISINGH: We started sometime ago looking at extracts of serum and found similar results; then we went back and extracted the same particles with DCM. Thus we knew how much DCM extractable mutagenic activity there should be on a certain mass of particles. We mixed the total DCM extract with serum, and the activityent right down to what it was when we incubated serum with the particles. So it looked as though serum was decreasing the activity, either through binding or other means. We couldn't measure it, however, and we had considerable difficulty in releasing that activity although we have tried with solvents, columns, and proteases. Do you have anything like that and have you combined extracts with serum?

T. CHAN: I haven't done that experiment yet. There is a paper being released showing that BaP can bind the lipid protein of the serum fractions. The lipid protein may thus be a serum fraction that will extract some mutagens out. But, we have to do the study before we say anything. What conclusions are you making from the fact that you are having such a rapid degradation of the mutagenic activity in the serum? The interpretation of what you have offered was that you feel the serum can extract the mutagenic activity.

J. HUISINGH: I think possibly this approach should be used to look at other test systems where mutagens bound with serum may be more detectable using cell culture systems.

MR. GEEM: Is there also a possibility that there may be some detoxification forces going on in the serum?

J. SIAK: No, when doing serum culture, using serum medium, you have serum there already.

L. SCHECHTMAN: In tissue culture systems, such as the serum hamster embryo system, we have used fetal calf serum to extract whatever mutagenic or transforming activity might be found using mutagenic compounds such as benz(a)-pyrene. We find that with continued incubation of BaP in fetal calf serum at 37 degrees over a 24 to 48 hour period one can, in fact, introduce as much as 10 to 30 micrograms of BaP per ml of pure fetal bovine serum. When that same serum is used as the serum supplement to growth medium to incubate the serum hamster embryo cells, one can change those cells to morphically transformed phenotypes. The serum serving as a "solubilizer" of the polycyclic hydrocarbon may be working as a double-edged sword not so much in terms of inactivating but making the carcinogen more bioavailable to the target cell system.

J. HUISINGH: I think that is an important point. We have not yet incubated these particles with serum and then introduced them to cell culture systems. I don't know whether General Motors has done that experiment or not, but I think that would help address the question. My assumption was that the serum was not inactivating but was simply binding the mutagens in such a way that they were not available to the bacteria and that is why we had attempted to release them or extract them back. I think it is just a matter of technique; we have not yet found the right method. We have had trouble with emulsions forming when we do extractions and proteas, although they might break up the proteins and create more histidine which interferes with the assay so we haven't found the right method yet.

D. CHOUDBURY: We observed that in extracting fiberglass splinters containing airborne particulates, a polar solvent like methanol extracts a lot more mass. We extracted the filters with benzene first and if the extraction is carried out for six hours almost 94-95 percent of it is extracted. We then took a series of these extracted filters and extracted them further with acidified and basic methanol and found that these methanol extracts had much more of the mass than any other solvent, but the mutagenic activity was very, very low. My assumption is that perhaps there is a lot of organic material in there, but the TLC plate shows that there isn't any organic. It is very highly polar.

R. YASBIN: Microbiologists have known for some time that even things like antibiotics when mixed with serum have great difficulty interfering with the processes in gram negative organisms. They are not detoxified; they are just bound, and I don't think detoxification is an explanation of what is happening with the serum. Probably, at least relating to what we have learned with antibiotics, they are bound and not allowed to get through the gram negative wall or membrane.

J. HUISINGH: I think that is an important point that probably should be tested. You are suggesting that serum can bind materials and be less available to bacteria, and from Dr. Schechtman's statements, it may make them more available to the mammalian cells. To draw conclusions about these serum extraction experiments, we really need to know a little more about what we are extracting.

MUTAGENIC ACTIVITY OF DIESEL EMISSION PARTICULATE EXTRACTS AND ISOLATION OF THE MUTAGENIC FRACTIONS

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ABSTRACT

Mutagenic activity in diesel emission particulate extracts was detected by the *Salmonella typhimurium*/microsome assay. Direct-acting mutagens as well as promutagens requiring metabolic activation were detected. The extracts were fractionated into acidic, basic, and neutral fractions, and the neutral fraction was chromatographed into seven subfractions. Differences in the mutagenic potency of these fractions and subfractions were determined by the *Salmonella* assay. Fractions containing as yet unidentified compounds, but not polynuclear aromatic hydrocarbons; were found to make a major contribution to mutagenic activity of the extracts.

INTRODUCTION

The carcinogenic activity of gasoline-powered automobile exhaust condensates is well known (1). Recently, organic extracts of diesel exhaust particulates have been shown to possess carcinogenic (2) and mutagenic (3) activities. However, the emission characteristics of diesel-powered vehicles are significantly different from those of gasoline-powered vehicles. A diesel automobile emits 30-50 times more particulates by mass than a comparable gasoline-powered automobile (4). Moreover, 90% of the diesel emission particulates are of respirable size, <1 μm (5), and high molecular weight organics are adsorbed onto them. The anticipated increase in use of diesel-powered light-duty vehicles will thus increase the automotive contribution to atmospheric particulate pollutants which are of public health concern. These considerations emphasize the importance of determining the toxicologic properties of organic compounds adsorbed onto the diesel exhaust particulates and identifying the constituents most responsible for the genotoxic effects.

Determination of the carcinogenic potential of an environmental sample by the conventional mouse-skin painting test, however, is time-consuming, expensive, and difficult. The microbial assay system introduced by Ames and his co-workers (6) is a fast, inexpensive, sensitive and reliable way to determine the mutagenic activity of a chemical, which has a strong qualitative correlation with its carcinogenic potential. Several histidine-requiring strains of *Salmonella typhimurium* are used for the assay.

Previous efforts to identify genotoxicants in diesel exhaust particulates have involved searching for compounds of known or suspected carcinogenicity, such as polynuclear aromatic hydrocarbons (PAH) (7), by appropriate analytical techniques --commonly gas chromatography-mass spectrometry. We assumed that the complex mixture of organic compounds in diesel particulate extracts is likely to contain mutagenic compounds other than commonly suspected ones. We therefore used the Ames test to detect mutagenicity in particulate extracts and to monitor the distribution of mutagenicity amoung various fractions of the extract. This communication presents the preliminary results of these assays, the fractionation of the extracts, and the isolation of fractions with relatively high mutagenic activity. Comprehensive chemical characterization of these fractions is under way.

EXPERIMENTAL

Sample Collection

Emission particulates from a diesel Volkswagen (VW) Rabbit and a diesel Mercedes 300-D were collected by a modification of the standard dilution tunnel technique (8) and using Pallflex T60A20 Teflon-coated glass fiber filters. The filters were extracted with dichloromethane for 24 h in a Soxhlet extractor. After filtration under vacuum through a 0.2-μm filter, the solvent was completely removed under vacuum with gentle heat. Details of the sample collection and extraction are described elsewhere in this Proceeding (8).

Fractionation of the Diesel Particulate Extracts

The first step in the fractionation was a liquid-liquid partitioning to separate the acidic, basic, and neutral compounds (Figure 1A). The sample was partitioned between dichloromethane and aqueous 0.1 N H_2SO_4 and aqueous 0.1 N NaOH to extract the bases and acids respectively in the aqueous phases. The basic and acidic compounds were subsequently back-extracted into dichloromethane after the pH of the aqueous phase was adjusted. When the acids and bases had been extracted, the organic layer was washed with water and dried over anhydrous sodium sulfate, and the solvent was evaporated to give the neutral fraction.

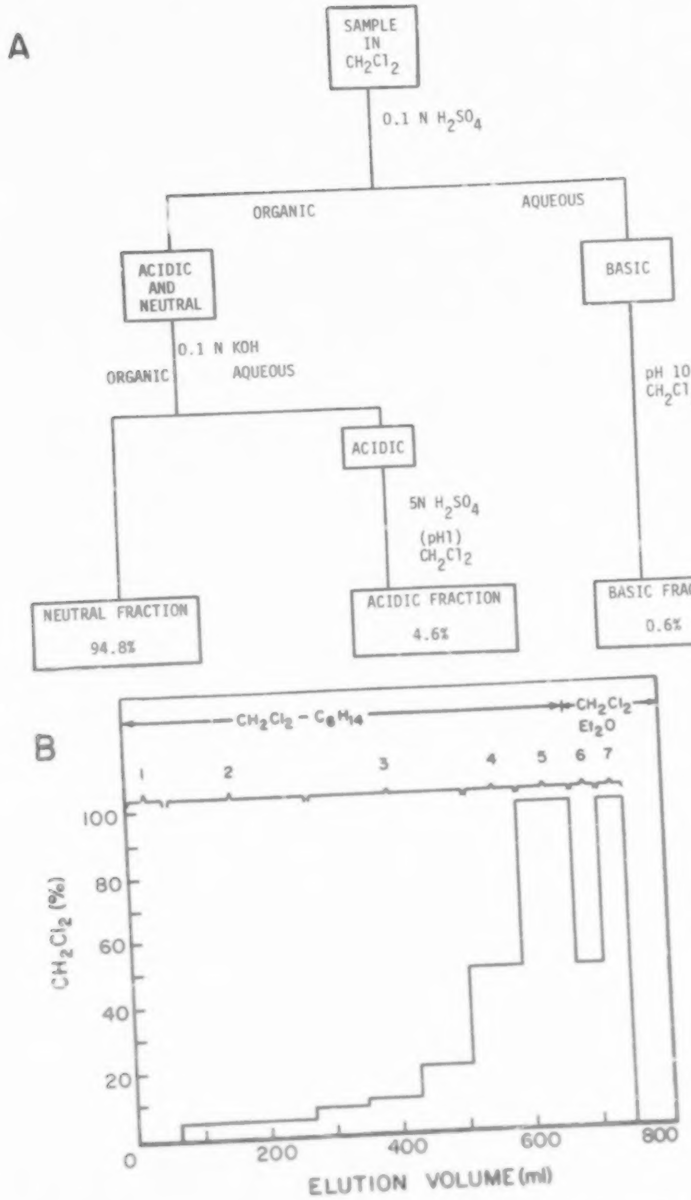

Figure 1. A) Fractionation of diesel emission particulate extracts. B) Elutant profile for chromatographic fractionation of neutral diesel particulate extract.

For the VW Rabbit sample the neutral fraction comprised approximately 94% of the extract by mass. It was further fractionated into seven subfractions by adsorption chromatography. Silica gel 60 (E. Merck, Germany) was washed with hexane and dichloromethane, dried and activated at 110°C for 12 h. The sample was then adsorbed onto 1 g of the freshly activated silica gel, which was added to the top of a chromatographic column (28 x 1 cm) packed with 9 g of dry, activated silica gel. A solvent gradient of hexane to dichloromethane to diethyl ether was used for elution. The elution profile is illustrated graphically in Figure 1B. The fractionation was monitored qualitatively by observing the fluorescence with an ultraviolet light.

Mutagenicity Assay

Histidine-requiring strains of Salmonella typhimurium (TA98, TA100, TA1535, TA1537, TA1538) were obtained from Dr. Bruce Ames of the University of California at Berkeley. The strains were routinely checked for histidine auxotrophy, deep rough character, and the presence of R factor (TA98 and TA100) as described by Ames et al. (6). The cells were grown in Difco bovine heart suspension. Liver homogeneate (S-9) from Aroclor 1254-induced male Sprague Dawley rats was obtained from Litton Bionetics (Kensington, Md). We found 0.075 ml of this S-9 preparation per ml of S-9 mix to be optimum for promoting optimal mutagenesis in TA98 by benzo(a)-pyrene (BaP) and 2-aminofluorene (2AF). This concentration was used in all subsequent experiments. Tests of diesel extract samples with lower or higher concentrations of S-9 did not increase the mutagenesis.

Each tester strain was routinely checked in the presence and absence of S-9 for optimal mutagenesis as follows:

TA98: BaP, 5 µg; 2AF, 2.5 µg; and 4-nitroquinoline-N-oxide (4NQO), 0.5 µg.

TA100: BaP, 5 µg, and 4NQO, 0.5 µg.
TA1535: N-methyl-N-nitro-N-nitrosoguanidine, 10 µg.
TA1537: 9-aminoacridine, 25 µg.
TA1538: 2AF, 2.5 µg.

A diesel particulate extract sample from a Nissan van was used with every set of experiments to check the consistency and reproducibility of the mutagenic response of each tester strain.

Each extract sample was dissolved in dimethyl sulfoxide and examined in the presence and absence of the metabolic activation system (S-9) in a dose response fashion (5-8 doses/tester strain. Revertant colonies were counted with a Biotran II, model C111 automated colony counter (New Brunswick Scientific Co., New Brunswick, NJ). Duplicate plates were run except for the subfractions (where the quantity of sample was insufficient), and the count of revertants was averaged. Each experiment was repeated at least once. All data were corrected for the spontaneous background reversion rate observed with each tester strain. A blank glass fiber filter was also extracted, and the extract was assayed for mutagenic response.

Solvents and Reagents

All solvents used for extraction and chemical fractionation were glass distilled,ultraviolet grade (Burdick and Jackson Inc., Muskegon, MI). Benzo(a)pyrene (gold label) and 2AF were obtained from Aldrich Chemical Company (Milwaukee, WI, 4NQO from ICN Pharmaceuticals (Plainview, N.Y.), and 9-aminoacridine and dimethylsulfoxide from Sigma Chemical Company (St. Louis, Mo).

RESULTS AND DISCUSSION

Extract of diesel exhaust particulates from the VW Rabbit showed positive mutagenic response to all five tester strains. The results with TA98, TA100, and TA1538 are shown in Figure 2. The responses with TA1535 and TA1537 were rather low. The highest response was with TA100. Significant mutagenic activity was observed with all five strains in the absence of the metabolic activation system (S-9), indicating the presence of direct-acting mutagens in the extract. Incorporation of S-9 lowered the mutagenic activity except in the case of TA1538. Similar deactivation of some direct-acting mutagens in the presence of S-9 has been reported before (9). The positive response with all strains also indicates that the mutagens in the extract presumably act by both base-pair substitution and frameshift mutation. An extract of Mercedes 300-D exhaust particulates also showed a high response with TA98 and TA100 (Fig. 3). The activity without S-9 was again higher than with S-9. The crude extracts were tested in duplicate at five concentrations in the range of 20-800 μg/plate. The number of revertants/plate (with background correction) was plotted against the dose (μg/plate), and slope of the linear part of the induction curve was determined by the least squares method. This slope was assumed to reflect the specific activity (revertants/μg). The results showed that specific activity of the Mercedes sample was somewhat higher than the Rabbit sample. In general, we found that TA98 and TA100 are good general indicators of mutagenicity of these samples. These two strains were therefore used to screen fractions of the extracts.

The extract was first fractionated into acidic, basic, and neutral fractions (Figure 1B). Some emulsion was formed during extraction with base and acid solutions. Use of dilute H_2SO_4 and NaOH solutions (0.1 N) in partitioning helped to

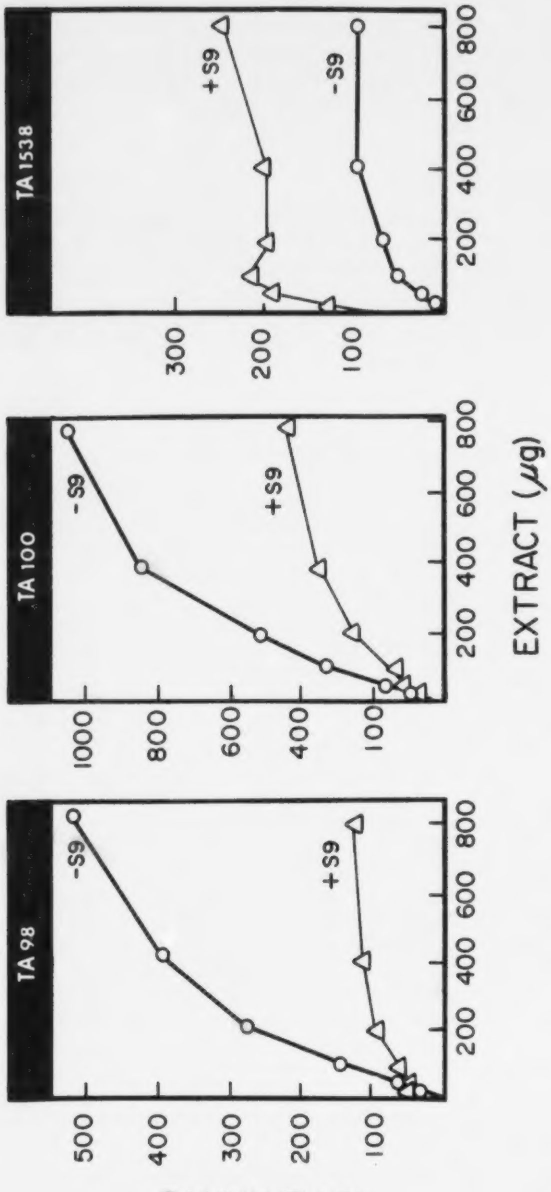

Figure 2. Mutation caused by diesel particulate extract (VW Rabbit).

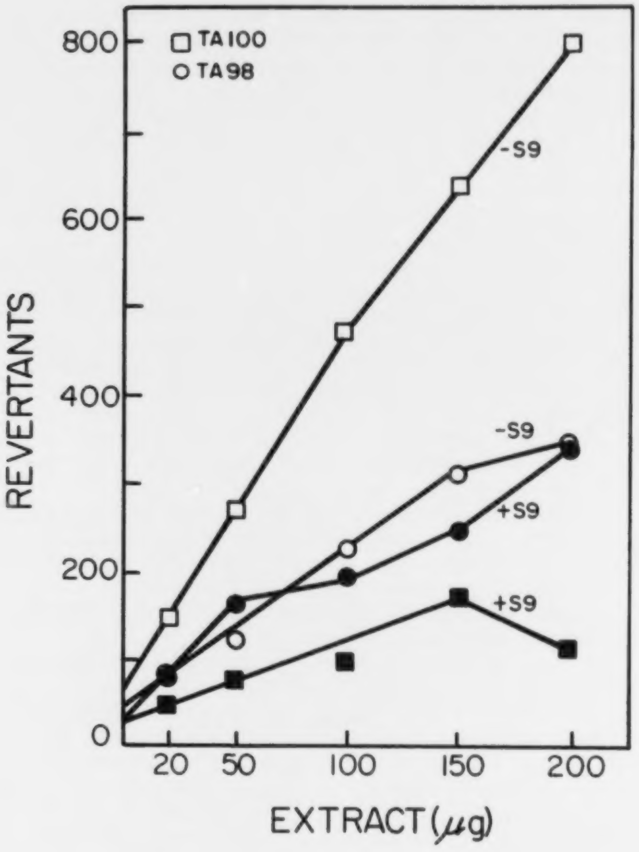

Figure 3. Mutagenic activity of diesel particulate extract (Mercedes 300-D).

reduce this problem. The extracts did not dissolve completely in ether, which could have minimized the emulsion problem.

The specific activities of the three fractions and the crude extract are shown in Figure 4. The acidic fraction had the highest specific activity, which was significantly higher in the absence than in the presence of S-9. The basic fraction, 0.5% by mass of the total, showed enhanced activity in the presence of S-9. The neutral fraction showed somewhat higher mutagenic response in the presence of S-9 than in its absence; the difference was smaller than for the crude extract. The results suggest that much of the direct-acting mutagens in the crude extract are acidic. Since the neutral fraction was the major fraction (95%) by mass, it was expected to contain most of the mutagenic compounds, including PAHs. We therefore developed an adsorption chromatographic system to subfractionate the neutral fraction.

Since the extracts are not soluble in hexane and since application of the sample in a polar solvent such as dichloromethane would result in loss of chromatographic resolution, we decided to apply the sample preadsorbed to a small amount of silica gel, which could be added to the top of a column containing dry, activated silica gel. This method of sample application is simpler than the one described by Rodriguez (10). The elution volume of the PAH fraction was determined by the elution volumes of fluorene and coronene, chromatographed under identical conditions.

The elution was started with hexane to carry off the paraffinic materials (fraction 1), and 70 ml of eluate was collected. The elutant was changed to 5% dichloromethane in hexane when fluorescent materials, observed under longwave UV, reached the bottom of the column. This fluorescent band was the PAH fraction (fraction 2). The elutant was gradually changed to 100% dichloromethane in several steps and finally to 100% diethyl ether (Figure 1B). Final elution with diethyl ether was essential to elute some highly adsorbing compounds.

Seven fractions were collected. The bulk of the solvent from each fraction was evaporated on a steam bath. The concentrated residue was quantitatively transferred to small, tared, screw-cap test tubes, dried under a gentle stream of nitrogen, and weighed. In the VW Rabbit sample the percentage of various fractions by mass was: fraction 1, 72.9%; fraction 2, 6.0%; fraction 3, 2.1%; fraction 4, 3.8%; fraction 5, 4.8%; fraction 6, 8.6%; and fraction 7, 1.8%.

All subfractions were assayed with strains TA98 and TA100. The tests were run at six to eight concentrations in the range of 2-100 µg/plate. Direct-acting mutagens were detected in subfractions 3-7. Subfraction 4 had the highest specific activity (Figure 5), as detected by both TA98 and TA100. The activity of subfractions 3 and 5 was enhanced in the presence of S-9, particularly with the strain TA100.

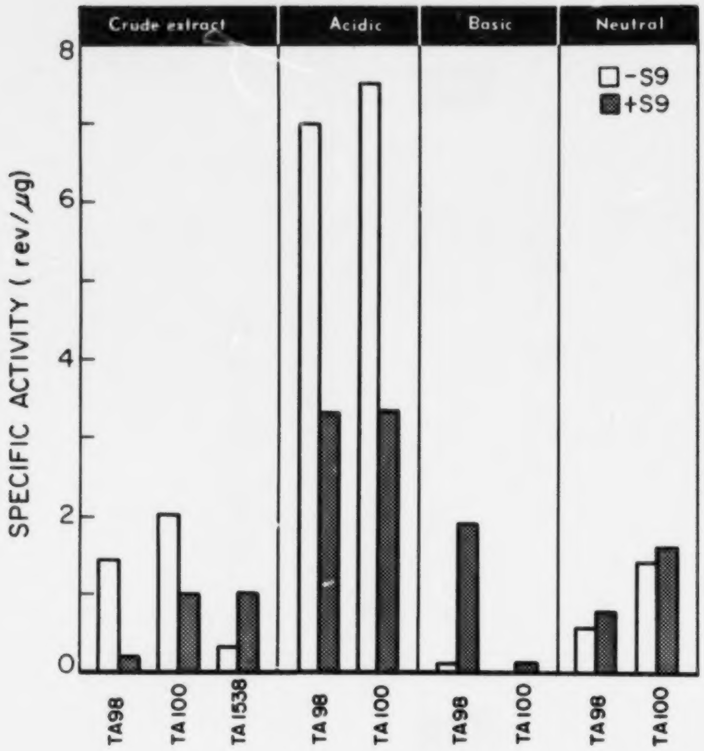

Figure 4. Mutagenicity of diesel particulate extract and its fractions (VW Rabbit).

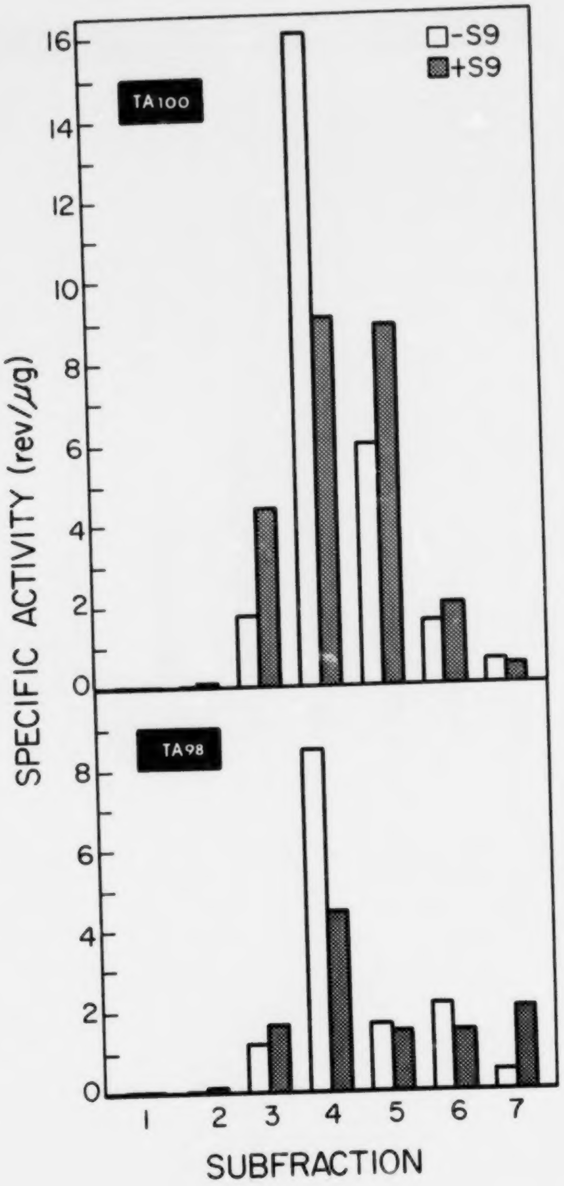

Figure 5. Distribution of mutagenicity among subfractions of neutral diesel particulate extract (VW Rabbit).

The paraffinic subfraction 1 showed no detectable activity, the PAH subfraction 2 showed marginal activity only in the presence of S-9. These results demonstrate that the objective of using the Salmonella assay and chemical fractionation to identify mutagenic fractions in the extracts has been achieved.

Since the paraffinic subfraction 1 is probably of little toxicological significance, we are ignoring it for the time being in our chemical characterization work. Subfraction 4 and the entire acid fraction will receive high emphasis for structural characterization; subfractions 3, 5, and 6 will also be characterized. Some subfractions will be further fractionated by high performance liquid chromatography for additional concentration of active components and to facilitate the identification of mutagenic compounds. Although the PAH subfraction 2 showed only marginal activity, many members of this class are recognized carcinogens and mutagens. Consequently we have undertaken a complete characterization of this fraction too. Preliminary results are described elsewhere in this Proceeding (11). No non-PAH compound was detected in this fraction by mass spectrometry, indicating clean separation of PAHs from paraffins and other classes of compounds.

Our results show the feasibility of the Ames test for detecting activity in diesel particulate extracts and various chemical subfractions of these extracts. Mutagens of various chemical classes can thus be separated from non-mutagens and weak mutagens, thereby facilitating chemical characterization. Also the relative contribution of known classes of mutagens (e.g. PAHs) compared to unknown ones in a complex mixture can be evaluated. We have used this test previously to isolate airborne mutagens (12), and it should be applicable to other complex environmental samples as well.

ACKNOWLEDGEMENT

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General Discussion

D. HOFFMANN: Your slides were the first I had seen of all the fractionation studies which showed that through fractionation you lose 20 percent of the whole tar, neutral, basic etc. Did you ever try the freeze-drying water layer and testing it? We have obtained mutagenicity with tobacco tar which was very surprising. We found activity when we took the water layer and freeze-dried it.

D. CHOUDHURY: I have not tried that. I think that 20 percent is primarily because there is a lot of water soluble material which is staying in the water layer and so far we haven't looked at that. I think your guess is right, and I tend to agree with that.

I. ALFHEIM: When you say you have a 20 percent loss, is that of the total extractable material, or is it a 20 percent loss of mutagenicity during the fractionation?

D. CHOUDHURY: That is the material by weight.

I. ALFHEIM: Did you know the recovery of the mutagenic activity of this fractionation?

D. CHOUDHURY: We haven't tried that, and in fact, I am not sure if one can actually do that. As you keep on fractionating this crude extract, you do get all kinds of senergisms and antagonisms coming into play and I am not sure if you can combine the activity and compare with the total. Some people have tried to do that and they claim they have done it quite successfully.

I. ALFHEIM: We do it on ambient air samples. When we combine the different fractions and test it again, we find the same as when we add the testing results from separate fractions.

D. CHOUDHURY: You mean the combining of different fractions together and then testing?

I. ALFHEIM: Yes, but they still seek the percentage loss during a procedure quite similar to this. That is why I asked if this was from different samples?

D. CHOUDHURY: We haven't done that, but I don't think there is actually any artifact during these fractionations. What you say would indicate that during fractionation, there is probably some change in the chemical character of this compound. That would perhaps occur. You lose the biological activities.

I. ALFHEIM: I was asking if there is any degradation?

D. CHOUDHURY: I believe there is some degradation but I do not have proof of that so far.

MUTAGENICITY STUDIES ON DIESEL PARTICLES AND PARTICULATE EXTRACTS

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ABSTRACT

Short-term mutagenicity assays by employing both different genetic end points and organisms treated with several methodologies have been developed on particles and DCM-extracted fractions obtained from a naturally aspirated swirl chamber high speed european diesel engine as a preliminary approach to the definition of the potential negative health effects on human population exposed to diesel emissions.

The *in vitro* methodologies have been based on the induction of: (i) reverse mutation on *S. typhimurium* TA1535, TA1537, TA1538, TA98, and TA100 strains; (ii) forward mutation on the yeast *S. pombe* P1 strain; (iii) mitotic gene conversion on the yeast *S. cerevisiae* D4 strain; (iv) unscheduled DNA synthesis (UDS) on human heteroploid EUE cell line.

The results so far obtained have shown the direct mutagenic activity of the particles when tested *in vitro* on *Salmonella* TA98 strain; this activity might be *possibly* due to the active fractions TRN and OXY which have resulted mutagenic on *Salmonella*.

The OXY fraction has been demonstrated to be able to produce forward mutation and mitotic gene conversions in two different yeast systems. The present *in vitro* data confirm those obtained by other AA and *complete them*, by showing the direct mutagenic activity of particles and the genotoxicity of the OXY fraction in other genetic systems not analyzed by other AA.

Preliminary data in our laboratories have been obtained on two heavy duty (sample A) and light duty (sample B) diesel engine extracts obtained from EPA: mutagenicity *in vitro* assays on *Salmonella* have confirmed the EPA *previously demonstrated activity* of these extracts. The Sample A diesel extract has been also tested for the induction of unscheduled DNA synthesis on human cells: the fraction has been found negative, but it inhibits the normal ^{3}H-TdR incorporation of the human cells.

The future plan of this project intends to develop other *in vitro* mutagenicity assays which analyze the induction of *other* genotoxic end points (gene-mutation in hamster cells, UDS in human cells, chromosome aberrations in human lymphocytes) and short term *in vivo* mutagenicity assays on mice and/or rats treated by *different* ways with particles or active particulate extract fractions (liver and lung tests with yeast; urine and serum tests with *Salmonella*; chromosome aberrations on bone marrow cells).

INTRODUCTION

Several AA (5,11,12,14) have recently shown that short-term mutagenicity *in vitro* assay, such as the *Salmonella*-microsome plate *test*, represents a suitable *biological* analytical procedure for the evaluation of the mutagenic activity of extractable organic compounds obtained from particles produced by different types of combustions (5,12) including different types of diesel engines (11): this approach allows both the identification of those fractions of chemicals produced during the combustions process which possess mutagenic activity and which could be further selected for chemical identification studies and for the evaluation of potential carcinogenic risk of diesel engine emissions due to the high positive correlation which has been observed between mutagenic and carcinogenic compounds (20,22,23,24, 25).

These results have stimulated the interest of public authorities and of automobile manufacturers in a possible negative health effect which could be the result of the exposure of human popoulation to diesel engine exhaust emissions, taking into account also the most probably future penetration of the market by diesel vehicles.

This consideration has prompted the development of chemical and mutagenic/carcinogenic researches on diesel engine particulate extract fractions with the aim of either identifying the combustions conditions on which it might depend the formation of mutagenic compounds, and either to evaluate, by applying the most appropriate short-term in vitro and in vivo mutagenicity assays, the potential carcinogenic and genotoxic risk to human population as a consequence of the exposure to diesel engine exhaust emissions, thus reducing, and possibly avoiding, a series of long-term carcinogenic animal assays.

Short-term in vitro mutagenicity assays which employ different biological systems (from bacteria to animals) (17), on which genetic end points of different molecular nature (gene mutation and chromosomal mutation) and of a different relevance for the evaluation of the genetic/carcinogenic risk represent only the possible rationale for identifying the highly genotoxic and potential carcinogenic fractions of chemicals present on the surface of the particulate and produced in a diesel engine combustion chamber.

More relevant however for the present interest is the use of appropriate in vivo methodologies which make use of microbial genetic systems and small rodents: these can be employed to confirm in vivo (in the mice) the mutagenic activity of the particles per se or of those fractions which have been identified as mutagenic after several in vitro experiments on different organisms. These methodologies, of the type of the host mediated assay (3,9) have been recently improved for their sensitivity (3): it is, therefore, possible to assess the presence or the in vivo formation of active metabolites in the liver, or in other organs (lung), by a direct assay, or to assess the presence of active metabolites in organic fluids, as the urines or the serum, taken from animals treated with diesel particles or their extracts. Other genetic end points, such as the chromosome mutation induction, may also be studied in vivo, in the somatic cells of treated animals (17), or in vitro, on human lymphocytes (17).

In the present paper we report the preliminary results obtained by in vitro mutagenicity experiments on the particles and four particulate DCM-extract fractions obtained from a naturally aspirated high speed swirl chamber european diesel engine (4); the assays have been developed on *Salmonella* five strains (TA1535, TA1537, TA1538, TA98, and TA100) (2), and two yeasts, namely *S. pombe* (P1 strain) (3) and *S. cerevisiae* (D4 strain) (15,16) which have allowed the evaluation of the potential activity of the samples for the production of gene-mutation in prokaryotic and eukaryotic cells and of the gene-conversion in a diploid cell.

Analyses have been made also on two EPA heavy duty (sample A) and light duty (sample B) diesel engine DCM total extracts with *Salmonella* 5 strains, by varying the amount of S-9 mix and for the induction of UDS (unscheduled DNA synthesis) on a human cell lines, an *in vitro* test that we want to apply to other samples of diesel engine exhausts emissions.

Further development of this work will make use of several *in vivo* methodologies, as those reported in figs. 1 and 2.

MATERIALS AND METHODS

I. *Genetic systems*: Figs 1 and 2 report the battery of short-term *in vitro* and *in vivo* tests for the evaluation of mutagenic potential of diesel engine particles and particulate extracts which we intend to use in our project. In the present paper the results of only the *in vitro* test 1, 2, 3 and 4 have been reported.

I.A *Salmonella reverse mutation test*: The method described by B. N. Ames et al. (2) has been used; in all experiments 500 ml of S-9 mix per plate was employed. In the experiments reported in Table 3 and Fig. 3 different amounts of S-9 mix were incorporated into the plates.

I.B *S. pombe forward mutation tests*: Yeast cell gene mutation test with *Schizosacharomyces pombe* is based on the treatment of a haploid double mutant strain (P1) of the genotype *Spade 6-60, rad 10-198, h*. It allows the evaluation of the frequency of forward gene mutations induced by a chemical in five different genetic loci located on the three chromosomes of this yeast. The strain employed consists of purple colonies (on complete medium), and the induced mutants consist of white colonies: they are of the genotype *ade 6-60, adex, rad 10-198*, and contain a second *ade x* mutation which has occurred in one of the following 5 genetic loci, namely, *ade 1, ade 3, ade 4, ade 5, ade 9* (10). Forward mutations induced by chemicals in each one of these 5 loci have been shown to consist of basepair substitutions as well as insertion/deletion base-pairs (10): 96 chemicals of different molecular nature have been so far tested in this mutational assay and 54 of them have been found positive. These include 38 direct acting mutagens and 16 promutagens (18).

I.C *S. cerevisiae gene conversion test*: Yeast cell mitotic *gene-conversion (mitotic intragene-recombination) test* in *Saccharomyces cerevisiae* is based on the treatment of *a diploid multimutant strain* (D4) of the genotype *α/a, gal2/+, ade 2-2/ade 2-1, trp 5-12/trp 5-27, leu 1/+.* It *allows the evaluation of the frequency of gene conversions* in the *ade 2* and *trp 5* loci, located on different *chromosomes of the yeast* induced by chemicals. Gene-conversions *ade 2* and *trp 5* have been shown to be sensitive *to hundreds of direct* and indirect acting mutagenic chemicals (16).

I.D *EUE cell line UDS test*: EUE human cells have been used *in the DNA repair test* which allows the detection of the ability of a chemical to stimulate the unscheduled DNA repair syntehsis (1,6,19). DNA repair is not a direct demonstration of the induction of a genetic effect; this process, however, is related to mutational as well to carcinogenic events (6) and the test allows the detection of many carcinogens (19).

2. *Methodologies*. The *in vitro* methodologies employed in *the present study do include* the treatment of the cells in the plates *(Salmonella)* or in liquid suspensions (yeast cells *and human cells*). Yeast cells were treated for several hours during their growth, and the general methodology was that used in several other studies already described (15). EUE human cells were seeded in each of 50 mm petri dishes, containing 4 coverslips and incubated with Dulbecco's modified Eagle's Minimal Essential Medium + 8% of calf serum at 37° C for 24 hours. After washing with Hank's balanced salt solution containing 20 mM HEPES buffer (HBSSH), the cells were treated with known concentations of chemicals under analysis, at 37° C for 1 hour, which were dissolved in small volumes of DMSO and then diluted with HBSSH. At the end of treatments, cells were washed 3 times with HBSSH incubated with fresh medium, or fresh medium +5 mM solution of Hydroxyurea (HU). After 15 min., all cultures received 10 µCi/ml ^{3}H-TdR (15-25 Ci/mmoles) and were further incubated for 4 hours. The cultures on coverslips were washed twice with cold HBSSH and extracted with ice-cold 1 M

HCL for 20 min.; they were then washed twice with ice-cold ethanol/ethylether (1:1) and twice with ice-cold ethylether dried and incubated overnight with toluene 350 (Packard) at room temperature, in scintillation vials. After addition of 10 ml standard scintillation mixture, the samples were counted in a Packard Tri-Carb Spectometer. The in vitro methodologies (Salmonella, S. pombe, S. cerevisiae, EUE cells) have all made use of S-9 mix obtained from aroclor treated rats (liver) according to previously described methods (3).

3. Chemicals: Particles were collected from a naturally aspirated swirl chamber high speed european diesel engine,[1] according to the methods described by C. Bassoli et al. (4); particulate extract fractions PRF, ARM, TRN, and OXY were prepared by A. DiLorenzo et al. (8). Reference mutagenic chemicals such as methylmethane-sulfurate (MMS) and dimethylnitrosamine (DMNA), employed in the UDS test, and 9-amino acridine, sodium azide, 2-aminofluorene employed in the Salmonella test were all of pure analytical grade. Sample A (heavy duty) and Sample B (light duty) diesel engine particulate DCM total extracts have been provided by U.S. EPA.

4. Evaluation of data: The evaluation of the induced genetic effect has been based on the regression analysis according to the equation $y = a + bx$, where (y) is the induced frequency of the genetic effect under consideration, and (x) is the dose of the mutagen (mg/plates in the case of Salmonella and mg/ml in the case of the two yeasts); the coefficients (a) and (b) are calculated by means of the least squares method (7). For each set of data the correlation coefficient r has been also evaluated.

RESULTS

Sample A and Sample B diesel extracts obtained from EPA (TRN fractions) were tested on the five strains of Salmonella typhimurium with and without S-9 mix: the same samples have been distributed in several laboratories. The results, reported in Tables 1A, 1B, 2A, and 2B, are quantitatively similar to those obtained by other AA (11). The presence of S-9 mix in the plates reduces the toxic effects of extracts:

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[1] 4-cyl 2.5 l displacement Diesel engine tested under 1975 Federal Test Procedure - Urban Driving Dynanometer Schedule.

with the aim to evaluate the possibility that a reduction of lethal effect might allow more revertant colonies to grow, an experiment has been performed on the Sample B diesel extract with different amounts of S-9 mix: the results are reported in Table 3 (Fig. 3) and they show that S-9 mix does not influence the mutagenic potency of this sample.

Sample A diesel extract has been also tested for its ability to induce unscheduled DNA syntehsis in human cells (EUE); this test informs us about the direct reactivity of a chemical under analysis towards the DNA, by evaluating the repair synthesis of DNA which might follow the induction of some type of damage to the DNA structure.

The results of this assay are reported in Tables 4A, 4B, 4C (Fig. 4); reference compounds such as MMS (Tab. 5B) and DMNA (Tab. 5A) have also included in this assay: concentrations below 1 mg/ml of Sample A extract in absence of S-9 mix do not stimulate unscheduled DNA synthesis, nor they show toxic effect (Tab. 4A); when more concentrated solutions have been tested these have been found highly toxic for normal ^{3}H-TdR incorporation (Tables 4B, and 4C and Fig. 4); on the contrary reference mutagenis either direct (MMS) or indirect (DMN) show a relative lower toxic effect and an increase of the ^{3}HTdR incorporation in the presence of HU (Tables 5A and 5B). By the present analysis it is possible to consider this diesel extract fraction as able to interfere with DNA replication, thus resembling the classical DNA inhibitors FUdR and BUdR (1), but, in any case there is an indication of a stimulation of an unscheduled DNA synthesis.

PRF, ARM, TRN and OXY particulate extract fractions have been tested on Salmonella, S. pombe, and S. cerevisiae, by the in vitro methodology. PRF and ARM fractions resulted negative in all test systems (Tables 6A, 6B, 7A, 7B, 8A, 8B); on the contrary TRN was mutagenic on TA1537, TA1538, TA98, and TA100 strains of Salmonella (Table 6C): this fraction resulted to contain direct mutagenic chemicals, although the presence of the metabolic system showed some increase of activity (Fig. 5). TRN was not mutagenic for the yeast S. pombe, and it did not induce mitotic gene conversions in S. cerevisiae (Tables 7C and 8C).

The OXY fraction was found positive in all three microbial systems (Tables 6D, 7D, and 8D). On Salmonella strains the higher tested dose produced a relevant toxicity (2,000 mg/plate); also for this fraction the presence of S-9 mix increased the mutagenic activity (Fig. 6). On S. pombe the OXY fraction was mutagenic (Table 7D) without S-9 mix, and the presence of the metabolizing system inhibited completely its mutagenic effect (Fig. 7). On S. cerevisiae the OXY fraction was also found mutagenic in the direct assay (Table 8D), but some genotoxic activity was also observed when the

S-9 mix was present (Fig. 8). Particles collected from this european diesel engine were assayed for their complete mutagenic activity *in vitro*, on *Salmonella* TA98 strain.

In vitro diesel exhaust particles were mutagenic (Table 9) either in presence and in absence of S-9 mix: its mutagenic activity has been compared with one of TRN and OXY fractions on the same strain: the results are reported in Fig. 9.

These two fractions represent 3.16% a,d 10.81% respectively of the total amount of diesel particles.

When we compare the specific direct mutagenic activity of the particles and the TRN and OXY fractions (250 mg/plate) the following values of revertants/mg are obtained: particles = 0.74; TRN = 2.08, and OXY = 6.70. These values correspond to the concentration ratios between TRN or OXY and the particles. Further experiments will define better the quantitative comparison of the mutagenic content of particles and the DCM-extracted fractions.

Preliminary experiments have been developed on some of the test assay presented in Figs. 1 and 2 for a definition of the most appropriate methodological procedures to be used in the future.

DISCUSSION

The Ames test applied to diesel particulate extracts prepared by EPA has shown that two samples analyzed have produced in our laboratories comparable values of mutagenic activity with those obtained by EPA: the increase of S-9 mix amount to the plates does not influence the mutagenic activity of the Sample B extract. A test has been also performed for the evaluation of the induction of unscheduled DNA synthesis in a human heteroploid cell line grown in culture: the sample has resulted inactive at non toxic levels (below 1 mg/ml), whereas it is extremely toxic to cell DNA replication at levels above 1 mg/ml: at concentrations higher than 1 mg/ml diesel particulate extract inhibits the normal DNA replication, thus demonstrating a direct effect of this fraction on DNA of the type of that observed for well known DNA inhibitors, such as BUdR and FUdR (1). In the presence of S-9 mix at 1 mg/ml, which does not inibit the normal DNA replication, the fraction does not stimulate the unscheduled DNA synthesis.

In the studies developed on the particles and the four extracted fractions obtained from a naturally aspirated swirl chamber high speed euorpean diesel engine, the *Salmonella* test has demonstrated the mutagenic activity on TA98 strain of the crude particles when tested directly and of the TRN and OXY fractions: these results confirm also for this type of particulate extracts the previous data (11).

If the positive results obtained so far will be confirmed also by further experiments on other types of diesel engine particles, it would appear the possibility to operate on particles instead of particulate extracts.

The OXY fraction resulted the most active in *Salmonella* in the absence of a metabolic activation system, which confirms the presence in the extract of direct acting frameshift mutagens, active on TA1537, TA1538, TA100, and TA98; this fraction resulted toxic at a level range of 1,000-2,000 mg/plate.

The OXY fraction has resulted also genotoxic for the yeast *S. pombe* P1 (forward gene mutation system) and for the yeast *S. cerevisiae* D4 (mitotic gene conversion system), thus supporting the hypothesis that this fraction contains highly aspecific mutagenic chemicals, as they have been found positive in several short-term assays (26); the present data confirm and complete the results obtained recently by the EPA program.

Other methodologies of the type shown in Fig. 1 are in progress in our laboratories on components exhausted and on different fractions from diesel engine emissions, as well as from particles obtained from industrial and domestic burners with the same fuel as that supplied to the present diesel engine: these comparative values are of the extreme relevance for the interpretation of the quantitative potential mutagenic/carcinogenic risk of diesel emissions.

At the present it is still impossible to define the potential genotoxic risk for human generation posed by diesel engine exhaust emissions, on the base of *in vitro* mutagenicity experiments if there are no sufficient *in vivo* experiments; moreover all these studies should produce results to be used for a comparison with similar risk posed by other air contaminants present in the human environment.

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IN VITRO

1. Salmonella Reverse Mutation
2. S. pombe Forward Mutation
3. S. cerevisiae Gene Conversion
4. EUE cell line UDS
5. Hamster V79 cell Forward Mutation
6. Human Lymphocytes Chrom. aberrations

IN VIVO (mouse)

1. URINE TEST : Salmonella Rev. Mutation
2. SERUM TEST : Salmonella Rev. Mutation
3. LIVER TEST : S. pombe Forw. Mutation
4. LUNG TEST : S. pombe Forw. Mutation
5. BONE MARROW : Chromosome Mutation

Figure 1. Battery of Short-Term in Vitro an Vivo Tests for the Evaluation of Mutagenic Potential of Diesel Engine Particles and Particulate Extracts.

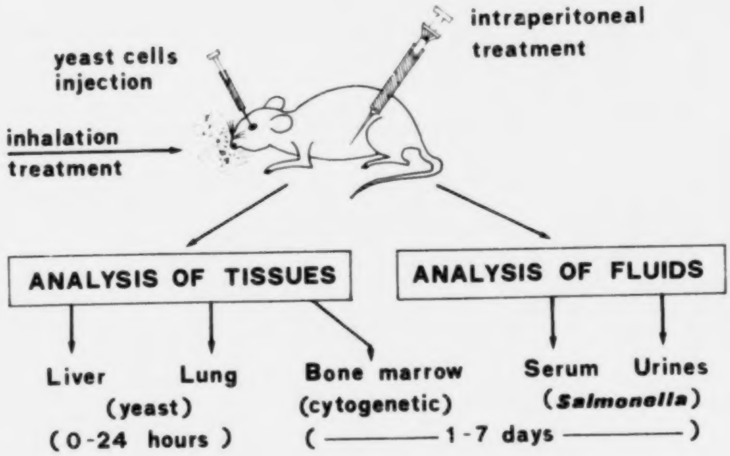

Figure 2. In Vivo Bioassay of Diesel Engine Particles.

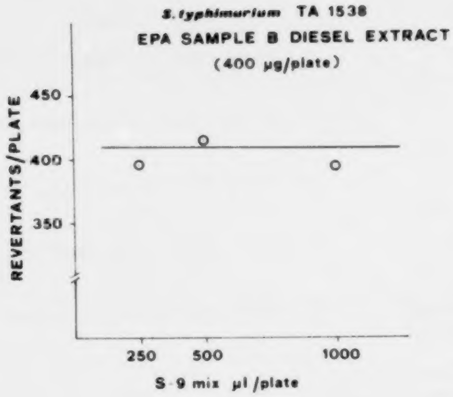

Figure 3. Reverse Gene Mutation Assay.

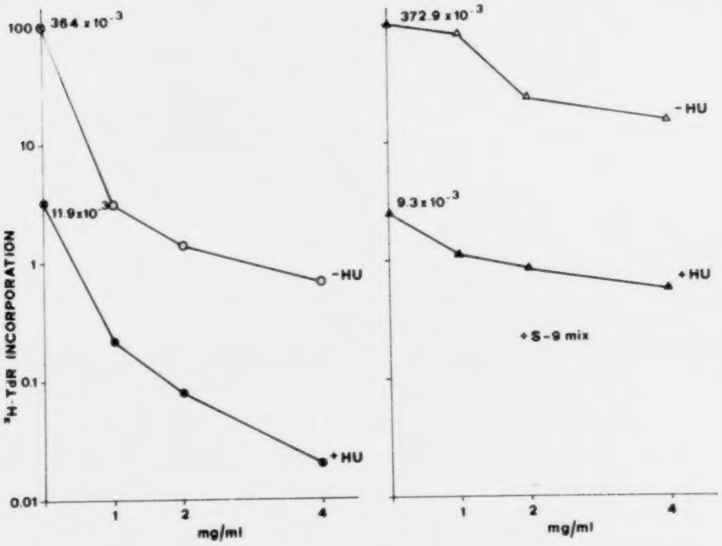

Figure 4. Unscheduled DNA Synthesis Assay.
(Sample A) Diesel Extract: EUE Cells

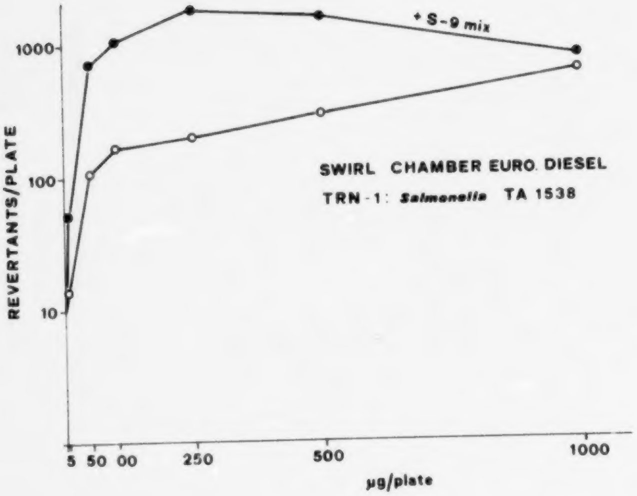

Figure 5. Reverse Gene Mutation Assay

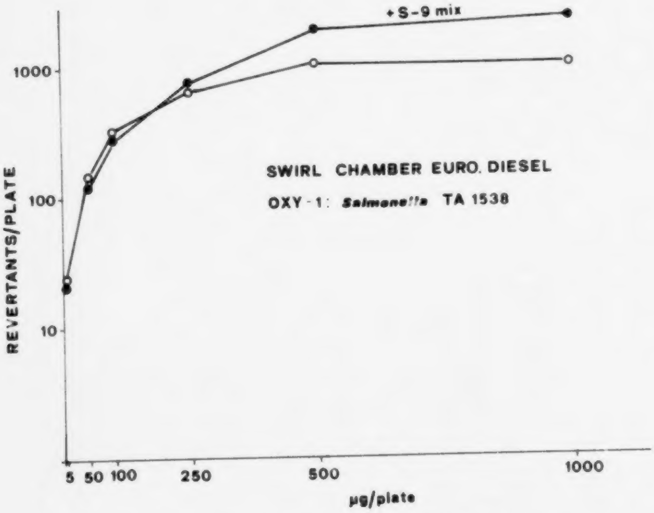

Figure 6. Reverse Gene Mutation Assay.

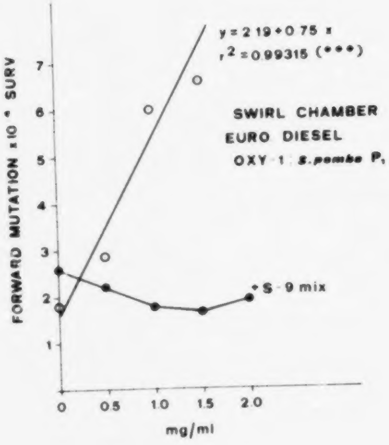

Figure 7. Forward Gene Mutation Assay

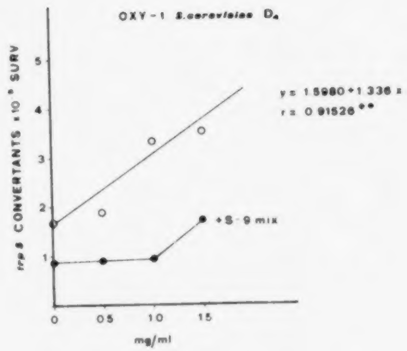

Figure 8. Mitotic Gene Conversion Assay
Swirl Chamber Euro.Diesel

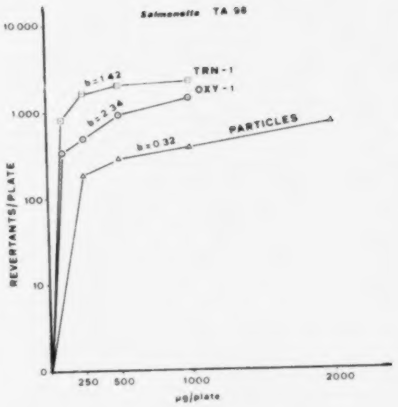

Figure 9. Reverse Gene Mutaticn Assay
Swirl Chamber Euro. Diesel.

TABLE 1A. MUTAGENICITY OF EPA SAMPLE A DIESEL EXTRACT DISSOLVED IN DMSO IN ABSENCE OF S-9 MIX ON SALMONELLA

Treatment	μg x Plate	TA1535	TA1537	TA1538	TA100	TA98
DMSO	0.2ml	19.5	5.5	14.5	74.5	34.0
Sample A	0	19.5	5.5	15.0	74.0	36.0
	50	19.0	11.5	14.5	98.0	41.0
Particulate	100	14.0	8.5	25.5	170.0	75.5
Extract	200	12.5	7.5	28.0	219.0	117.5
	400	13.5	10.5	42.0	335.0	175.5
	800	12.5	7.0	73.5	493.0	276.0
9-Amino Acridine	10	-	>1000	-	-	-
Sodium Azide	5	1290	-	-	1020	-

TABLE 1B. MUTAGENICITY OF EPA SAMPLE A DIESEL EXTRACT DISSOLVED IN DMSO IN PRESENCE OF S-9 MIX (500 L S-9 MIX ON SALMONELLA

Treatment	μg x Plate	TA1535	TA1537	TA1538	TA100	TA98
DMSO	0.2ml	19.5	5.5	14.5	14.5	35.0
Sample A	0	19.5	5.5	15.0	74.0	36.0
	50	18.5	7.5	18.5	95.5	35.0
Diesel Partic.	100	19.5	6.0	25.5	110.0	46.5
Extract	200	19.0	7.0	42.0	119.0	62.0
	400	18.0	4.0	69.5	147.0	94.0
	800	21.5	4.0	160.0	210.0	155.0
2-Amino Fluorene	5	-	-	530.0	435.0	765.0

TABLE 2A. MUTAGENICITY OF EPA SAMPLE B DIESEL EXTRACT DISSOLVED IN DMSO IN ABSENCE OF S-9 MIX ON SALMONELLA

Treatment	µg x Plate	TA1535	TA1537	TA1538	TA100	TA98
DMSO	0.2ml	18	5	16	79	32
Sample B	0	22	5	15	74	35
	50	14	7	40	293	144
Diesel	100	15	12	75	706	368
Extract	200	16	25	108	902	620
	400	22	24	133	1500	877
	800	26	30	TOXIC	2000	1042
9-Amino Acridine	10	-	>1000	-	-	-
Sodium Azide	5	1290	-	-	1020	-

TABLE 2B. MUTAGENICITY OF EPA SAMPLE B DIESEL EXTRACT DISSOLVED IN DMSO IN PRESENCE OF S-9 MIX (500 L S-9 MIX) ON SALMONELLA

Treatment	µg x Plate	TA1535	TA1537	TA1538	TA100	TA98
DMSO	0.2ml	18	5	16	79	32
Sample B	0	17	7	15	72	35
	50	23	11	54	161	88
Diesel	100	22	6	121	228	133
Extract	200	23	7	270	408	352
	400	54	13	425	641	636
	800	54	18	634	1100	850
2-Amino Fluorene	5	-	-	520	460	730

TABLE 3. MUTAGENICITY OF EPA SAMPLE B DIESEL EXTRACT DISSOLVED IN DMSO IN THE PRESENCE OF DIFFERENT AMOUNT OF S-9 MIX ON SALMONELLA

Treatment	µg x plate	S-9 mix 1 x plate	TA1538
Sample B	400	250	395
Diesel	400	500	416
Extract	400	1000	393

TABLE 4A. DNA REPAIR TEST (HUMAN CELLS) EPA SAMPLE A DIESEL EXTRACT

Dose	S-9	^{3}H-TdR Incorporation			^{3}H-TdR Incorporation HU-resistant	
		-HU		+HU		
µg/ml	Mix	cmp x 10^{-3} ± SE	%	cmp x 10^{-3} ± SE	%	Increase Over Control
-	-	783.7 ± 0.9	100	36.3 ± 0.9	4.63	1.00
83	-	925.2 ± 18.9	118	35.9 ± 0.8	3.88	0.83
250	-	538.2 ± 48.4	68	20.5 ± 1.6	3.81	0.82
830	-	797.5 ± 10.2	102	38.3 ± 8.0	4.86	1.04

TABLE 4B. DNA REPAIR TEST (HUMAN CELLS) EPA SAMPLE A DIESEL EXTRACT

Dose	S-9	^{3}H-TdR Incorporation				^{3}H-TdR Incorporation HU-resistant	
		-HU		+HU			Increase Over
µg/ml	Mix	cmp x 10^{-3}±SE	%	cmp x 10^{-3}±SE		%	Control
-	-	364.0 ± 19.0	100	11.9 ± 6.3		3.28	1.00
1	-	11.5 ± 1.6	3.2	0.8 ± 0.03		7.26	2.21
2	-	4.3 ± 0.5	1.2	0.3 ± 0.1		6.07	1.85
4	-	2.7 ± 0.03	0.7	0.1 ± 0.01		4.67	1.42
-	+	372.9 ± 18.0	100	9.3 ± 0.1		2.50	1.00
1	+	321.2 ± 5.2	86	4.1 ± 0.8		1.28	0.51
2	+	89.9 ± 1.1	24	3.3 ± 0.3		3.72	1.48
4	+	56.6 ± 5.3	15	2.2 ± 0.6		3.88	1.55

TABLE 4C. DNA REPAIR TEST (HUMAN CELLS) EPA SAMPLE A DIESEL EXTRACT

Dose	S-9	^{3}H-TdR Incorporation				^{3}H-TdR Incorporation HU-resistant	
		-HU		+HU			Increase Over
μg/ml	Mix	cmp x 10^{-3}±SE	%	cmp x 10^{-3}±SE		%	Control
-	-	1000 ± 0.0	100	30.9 ± 0.9		3.1	100
5	-	12.1 ± 5.6	1.2	3.6 ± 1.1		29.7	9.58
25	+	0.5 ± 0.6	0.05	0.2 ± 0.2		40.0	12.90

TABLE 5A. DNA REPAIR (HUMAN CELLS) COMPOUND: DIMETHYLNITROSAMINE (DMN)

Dose mM	S-9 Mix	^{3}H-TdR Incorporation -HU cmp x 10^{-3}±SE	%	^{3}H-TdR Incorporation +HU cmp x 10^{-3}±SE	^{3}H-TdR Incorporation HU-resistant %	Increase Over Control
0	+	565.5 ± 12.9	100.0	18.6 ± 1.7	3.3	1.0
200	+	183.7 ± 7.5	32.5	28.8 ± 3.0	15.7	4.8
300	+	252.6 ± 4.6	44.7	33.1 ± 3.5	13.1	4.0
400	+	126.3 ± 6.6	22.3	46.0 ± 5.4	36.4	11.1
400	+	336.2 ± 41.0	59.5	11.8 ± 1.8	3.5	1.1

TABLE 5B. DNA REPAIR (HUMAN CELLS) COMPOUND: METHYLMETHANESULFONATE (MMS)

Dose mM	S-9 Mix	^{3}H-TdR Incorporation			^{3}H-TdR Incorporation HU-resistant	
		-HU		+HU		
		cmp x $10^{-3} \pm$ SE	%	cmp x $10^{-3} \pm$ SE	%	Increase Over Control
0.0	-	452.0 ± 27.9	100.0	12.7 ± 0.7	2.8	1.0
0.5	-	413.1 ± 9.7	91.4	22.2 ± 1.3	5.4	1.9
1.0	-	374.5 ± 39.4	82.9	29.9 ± 0.2	8.0	2.9
2.0	-	270.6 ± 26.0	59.9	51.7 ± 0.1	19.1	6.8

TABLE 6A. MUTAGENICITY OF PRF-1 FRACTION FROM PARTICULATE EXTRACTS OF A NATURALLY ASPIRATED SWIRL CHAMBER EURO. DIESEL ENGINE ON SALMONELLA WITH AND WITHOUT S-9 MIX

Treatment	S-9 Mix	μg/plate	TA1535	TA1537	TA1538	TA100	TA98
DMSO	-	0.2ml	9	5	16	154	43
PRF	-	5	5	5	6	158	40
	-	50	7	3	12	147	41
	-	100	6	7	6	131	31
	-	250	6	3	2	100	33
	-	500	10	9	3	101	33
	-	1000	7	7	10	111	36
	-	5000	8	10	3	90	22
DMSO	+	0.2ml	10	7	12	137	30
PRF	+	5	8	2	7	130	36
	+	50	12	3	7	133	35
	+	100	5	3	6	134	34
	+	250	9	3	5	147	37
	+	500	5	9	7	149	19
	+	1000	10	6	9	172	14
	+	5000	7	3	8	165	34

TABLE 6B. MUTAGENICITY OF ARM-1 FRACTION FROM PARTICULATE EXTRACTS OF A NATURALLY ASPIRATED SWIRL CHAMBER EURO, DIESEL ENGINE ON SALMONELLA WITH AND WITHOUT S-9 MIX

Treatment	S-9 Mix	μg/plate	TA1535	TA1537	TA1538	TA100	TA98
DMSO	-	0.2ml	5	6	9	139	23
PRF	-	5	5	4	5	133	12
	-	50	9	10	4	144	26
	-	100	9	7	15	145	20
	-	250	7	11	7	144	19
	-	500	3	6	7	139	18
	-	1000	7	1	6	144	20
	-	3000	9	5	8	138	39
DMSO	+	0.2ml	10	8	9	122	22
PRF	+	5	7	2	9	136	16
	+	50	17	8	12	151	14
	+	100	6	4	9	152	30
	+	250	6	4	17	148	34
	+	500	8	9	11	170	30
	+	1000	11	8	28	195	34
	+	3000	13	17	30	200	57

TABLE 6C. MUTAGENICITY OF TRN-1 FRACTION FROM PARTICULATE EXTRACTS OF A NATURALLY ASPIRATED SWIRL CHAMBER EURO. DIESEL ENGINE ON SALMONELLA WITH AND WITHOUT S-9 MIX

Treatment	S-9 Mix	$\frac{\mu g}{plate}$ (x)	TA1535 (y)	TA1537 (y)	TA1538 (y)	TA100 (y)	TA98 (y)
DMSO	-	0.2ml	5	6	9	139	23
TRN	-	5	6	9	14	207	50
	-	50	15	40	110	570	190
	-	100	15	50	166	843	370
	-	250	12	92	200	1385	520
	-	500	11	170	300	2124	975
	-	1000	17	180	620	2420	1460
DMSO	+	0.2ml	10	8	8	122	22
TRN	+	5	12	20	54	247	60
	+	50	36	190	738	1320	430
	+	100	54	263	1060	1740	940
	+	250	55	450	1884	1950	2100
	+	500	19	232	1600	2150	2800
	+	1000	3 (TOXIC)	55 (TOXIC)	800 (TOXIC)	1340 (TOXIC)	3000

Regression Analysis

TA1537	-S-9	$y = 13.410 + 0.31662x$	$r = 0.99386$***
	+S-9	$y = 46.169 + 1.7288x$	$r = 0.96722$***
TA1538	-S-9	$y = 48.861 + 0.56534x$	$r = 0.98320$***
	+S-9	$y = 152.31 + 7.364x$	$r = 0.97323$***
TA100	-S-9	$y = 295.94 + 3.859x$	$r = 0.98356$***
	+S-9	$y = 513.60 + 6.9407x$	$r = 0.84305$*
TA98	-S-9	$y = 125.3 + 1.4230x$	$r = 0.98323$***
	-S-9	$y = 493.95 + 3.0942x$	$r = 0.80213$*

TABLE 6D. MUTAGENICITY OF OXY-1 FRACTION FROM PARTICULATE EXTRACTS OF A NATURALLY ASPIRATED SWIRL CHAMBER EURO. DIESEL ENGINE ON SALMONELLA WITH AND WITHOUT S-9 MIX

Treatment	S-9 Mix	µg/plate (x)	TA1535 (y)	TA1537 (y)	TA1538 (y)	TA100 (y)	TA98 (y)
DMSO	-	0.2ml	9	5	16	154	43
OXY	-	5	10	13	24	161	63
	-	50	12	62	150	431	403
	-	100	24	112	330	630	830
	-	250	22	264	661	1010	1680
	-	500	20	541	1052	1500	2120
	-	1000	17 (TOXIC)	291 (TOXIC)	1020 (TOXIC)	1770	2350 (TOXIC)
	-	2000				685 (TOXIC)	
DMSO	+	0.2ml	10	7	12	137	30
OXY	+	5	13	8	21	134	41
	+	50	18	25	125	332	228
	+	100	25	60	300	490	504
	+	250	66	175	762	880	1600
	+	500	63	381	1860	1284	3200
	+	1000	36	443 (TOXIC)	1130	2020	4120
	+	2000	8 (TOXIC)	81 (TOXIC)	491 (TOXIC)	1820	3600 (TOXIC)

Regression Analysis

TA1537	-S-9	$y = 5.7616 + 1.0635x$	$r = 0.99980$***
	+S-9	$y = 4.7539 + 0.75638x$	$r = 0.99748$***
TA1538	-S-9	$y = 56.084 + 2.0956x$	$r = 0.98993$***
	+S-9	$y = 0.40559 + 3.6722x$	$r = 0.99548$***
TA100	-S-9	$y = 246.25 + 2.6613x$	$r = 0.98167$***
	+S-9	$y = 193.19 + 2.3181x$	$r = 0.98595$***
TA98	-S-9	$y = 430.36 + 2.3499x$	$r = 0.89859$*
	+S-9	$y = 438.38 + 1.9597x$	$r = 0.96698$***

TABLE 7A. MUTAGENICITY OF PRF-1 FRACTION FROM PARTICULATE EXTRACTS OF A NATURALLY ASPIRATED SWIRL CHAMBER EURO. DIESEL ENGINE ON A FORWARD MUTATIONAL YEAST ASSAY (S. POMBE) WITH AND WITHOUT S-9 MIX

Treatment	mg/ml	S-9 Mix	No. Mutants: No. Colonies	Mutations Frequency x10^{-4}	Survival x10^{-2}
Control	0	-	2: 5500	0.36	100
PRF	0.47	-	1:27027	0.37	71
	0.94	-	1:15150	0.66	70
	1.88	-	2: 4761	0.42	79
Control	0	+	1:15870	0.63	100
PRF	0.47	+	1:27030	0.37	83
	0.94	+	1:13510	0.74	60
	1.88	+	1:17240	0.58	76

TABLE 7B. MUTAGENICITY OF ARM-1 FRACTION FROM PARTICULATE EXTRACTS OF A NATURALLY ASPIRATED SWIRL CHAMBER EURO. DIESEL ENGINE ON A FORWARD MUTATIONAL YEAST ASSAY (S. POMBE) WITH AND WITHOUT S-9 MIX

Treatment	mg/ml	S-9 Mix	No. Mutants: No. Colonies	Mutations Frequency x10^{-4}	Survival x10^{-2}
Control	0	-	4:28200	1.41	100
ARM	1.2	-	5:21675	2.30	77
	2.4	-	7:23625	2.96	83
	4.9	-	3:30750	0.97	100
Control	0	+	2:20550	0.97	100
ARM	1.2	+	4:30750	1.30	100
	2.4	+	6:26700	2.24	100
	4.9	+	7:25275	2.76	100

TABLE 7C. MUTAGENICITY OF TRN-1 FRACTION FROM PARTICULATE EXTRACTS OF A NATURALLY ASPIRATED SWIRL CHAMBER EURO. DIESEL ENGINE ON A FORWARD MUTATIONAL YEAST ASSAY (S. POMBE) WITH AND WITHOUT S-9 MIX

Treatment	mg/ml	S-9 Mix	No. Mutants: No. Colonies	Mutation Frequency x10^{-4}	Survival x10^{-2}
Control	0	-	6:27675 7:22725	2.62	100
TRN	0.5	-	5:12600 5:32400	3.54	63
	1.0	-	2: 7350 3:36675	1.76	60
	2.0	-	13:26025 5:37725	3.15	89
Control	0	+	8:17640 3:85760	2.44	100
TRN	0.5	+	10:28275 8:47100	2.61	79
	1.0	+	2:13050 2:18075	1.27	41
	2.0	+	5:23475 4:47925	1.47	68

TABLE 7D. MUTAGENICITY OF OXY-1 FRACTION FROM PARTICULATE EXTRACTS OF A NATURALLY ASPIRATED SWIRL CHAMBER EURO. DIESEL ENGINE ON A FORWARD MUTATIONAL YEAST ASSAY (S. POMBE) WITH AND WITHOUT S-9 MIX

Treatment	mg/ml (x)	S-9 Mix	No. Mutants: No. Colonies	Mutation Frequency $\times 10^{-4}$ (x)	Survival $\times 10^{-2}$
Control	0	-	5:33525 6:27675	1.82	100
OXY	0.5	-	10:48300 10:40500	2.88	100
	1.0	-	11:12900 2: 5775	6.00	29
	1.5	-	2: 3244	6.16	10
	2.0	-	TOXIC	----	TOXIC
Control	0	+	2:25425 8:17640	2.65	100
OXY	0.5	+	9:61800 11:34950	2.29	100
	1.0	+	3:37950 5:18300	1.76	92
	1.5	+	6:29550	1.69	100
	2.0	+	5:26040	1.92	100

Regression Analysis:

$y = 2.19 + 0.75x$

$r^2 = 0.99315$***

TABLE 8A. MUTAGENICITY OF PRF-1 FRACTION FROM PARTICULATE EXTRACTS OF A NATURALLY ASPIRATED SWIRL CHAMBER EURO. DIESEL ON A MITOTIC GENE CONVERSION (S. CEREVISIAE: D_4) WITH AND WITHOUT S-9 MIX

			Mitotic Gene Conversion Frequency $\times 10^{-5}$		
Treatment	mg/ml	S-9 Mix	ADE_2	TRP_5	Survival $\times 10^{-2}$
Control	0	-	1.75	1.25	100
PRF	1.2	-	1.61	1.24	100
	2.4	-	1.43	1.40	100
	4.9	-	1.19	1.13	100
Control	0	+	1.24	0.69	100
PRF	1.2	+	1.39	0.99	82
	2.4	+	0.82	0.58	100
	4.9	+	1.15	1.19	91

TABLE 8B. MUTAGENICITY OF ARM-1 FRACTION FROM PARTICULATE EXTRACTS OF A NATURALLY ASPIRATED SWIRL CHAMBER EURO. DIESEL ON A MITOTIC GENE CONVERSION (S. CEREVISIAE: D_4) WITH AND WITHOUT S-9 MIX

			Mitotic Gene Conversion Frequency $\times 10^{-5}$		
Treatment	mg/ml	S-9 Mix	ADE_2	TRP_5	Survival $\times 10^{-2}$
Control	0	-	1.75	1.25	100
ARM	0.47	-	1.62	0.90	100
	0.94	-	1.32	0.94	100
	1.88	-	1.38	0.84	100
Control	0	+	1.24	0.69	100
ARM	0.47	+	1.23	0.80	86
	0.94	+	1.31	0.62	82
	1.88	+	1.02	0.50	90

TABLE 8C. MUTAGENICITY OF TRN-1 FRACTION FROM PARTICULATE EXTRACTS OF A NATURALLY ASPIRATED SWIRL CHAMBER EURO. DIESEL ON A MITOTIC GENE CONVERSION (S. CEREVISIAE: D_4) WITH AND WITHOUT S-9 MIX

Treatment	mg/ml	S-9 Mix	Mitotic Gene Conversion Frequency $x10^{-5}$		Survival $x10^{-2}$
			ADE_2	TRP_5	
Control	0	-	2.51	1.74	100
TRN	0.5	-	2.64	1.55	100
	1.0	-	1.37	1.47	100
	2.0	-	2.11	1.38	88
Control	0	+	0.82	0.85	100
TRN	0.5	+	2.11	0.93	70
	1.0	+	1.32	1.05	77
	2.0	+	2.42	1.23	73

TABLE 8D. MUTAGENICITY OF OXY-1 FRACTION FROM PARTICULATE EXTRACTS OF A NATURALLY ASPIRATED SWIRL CHAMBER EURO. DIESEL ON A MITOTIC GENE CONVERSION (S. CEREVISIAE: D_4) WITH AND WITHOUT S-9 MIX

Treatment	mg/ml (x)	S-9 Mix	Mitotic Gene Conversion Frequency $x10^{-5}$		Survival $x10^{-2}$
			ADE_2	(y) TRP_5	
Control	0	-	2.51	1.74	100
OXY	0.5	-	1.76	1.83	83
	1.0	-	1.76	3.38	70
	1.5	-	3.10	3.45	34
Control	0	+	0.82	0.85	100
OXY	0.5	+	0.84	0.87	97
	1.0	+	1.03	0.93	100
	1.5	+	2.67	1.76	56

Regression analysis:

$y = 1.5980 + 1.336x$

$r = 0.91526^{***}$ (trp 5)

TABLE 9. IN VITRO MUTAGENICITY ON SALMONELLA TA98 OF PARTICLES COLLECTED FROM A NATURALLY ASPIRATED SWIRL CHAMBER EURO. DIESEL ENGINE

Dose mg/plate (x)	No. Revertants per plate (±S.E.)	
	- S-9 Mix (y)	+ S-9 Mix (y)
0	28.6 ± 4.0	45.3 ± 7.4
250	186.3 ± 13.0	153.6 ± 4.9
500	292.3 ± 3.2	248.0 ± 24.8
1000	398.6 ± 19.3	386.0 ± 32.1
2000	714.6 ± 27.5	698.0 ± 22.1

Regression analysis:

- S-9 $y = 82.475 + 0.3223\,x \quad r = 0.9882^{**}$

+ S-9 $y = 67.325 + 0.3185\,x \quad r = 0.99794^{**}$

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General Discussion

A. KOLBER: I notice as I extrapolate your data that for this fraction you get between two and three revertents per microgram, which is the kind of mutagenicity quantification that a lot of people get. I noticed from Dr. Chan's talk from General Motors, they obtained as much as 40 revertents per microgram in their neutral polar fraction. I wonder if either one of you would like to comment about the quantification. Why is one sample so highly mutagenic and another rather low? Is there any reason for this difference in quantification?

N. LOPRIENO: Our data shows an induction of mutation. I showed that one of the fractions was completely different from the other, but I don't really have an explanation.

J. SIAK: I will reply to the question. We follow all the activity we put in and then calculate all the activity from each fraction. We get around 85 to 90 percent of the activity we put into it before the fractionation scheme.

N. LOPRIENO: We have not completed all the work with regards to the total extract.

J. SIAK: The discrepancy may be due to the procedures used in your activation process. We avoid light and we don't expose to high temperature. We try to evaporate under pressure, or under vacuum, and try not to use diethyl ether. So I think for those doing fractionations they should consider those procedures to avoid the loss of activity.

N. LOPRIENO: As I stated, our first aim was to develop a small data set from a different system on just the one sample, and then the next step was to do very quantitative studies in order to correlate to the mutagenic potential with different test systems. The numerical data you saw are only that way we use to evaluate the positivity of themselves by the regulation analysis.

J. HUISINGH: Do you have any data yet on the V79 cell line?

N. LOPRIENO: No, I do not yet.

THE MUTAGENICITY OF DIESEL EXHAUST EXPOSED TO SMOG CHAMBER CONDITIONS AS SHOWN BY SALMONELLA TYPHIMURIUM

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ABSTRACT

Since previous work demonstrating the mutagenicity of particle-associated organics was performed using particles collected with laboratory dilution tunnels, this study explored the significance of ambient conditions through the use of a smog chamber. Diesel particles exposed within the Calspan Smog Chamber Facilities, Ashford, New York, were collected on Palfex T60A20 glass fiber filters, extracted in dichloromethane, solvent exchanged to dimethylsulfoxide and tested in the Ames *Salmonella typhimurium* plate incorporation assay. It was demonstrated that the mutagenic response of these exhaust organics depends upon the method of collection and is altered by certain ambient conditions. This study clearly demonstrates that the level of mutagenic activity is influenced by the presence of ozone. This study also demonstrated that the UV irradiation of a simple mixture of propylene, NO, NO_2, and SO_2 produced mutagenic moieties. Future experiments will continue to examine factors within ambient air that modify mutagenic activity.

INTRODUCTION

Most studies with diesel exhaust have not described what effects exposure to ambient-like conditions would have upon particle-associated organics, since previous studies have used dilution-tunnel methods for the collection of exhaust particles (1). This study exposed diesel exhaust to specific ambient-like conditions through the use of a smog chamber. The effect of these ambient conditions was measured by testing the particle-associated organics in the *Salmonella typhimurium* plate incorporation assay.

This study describes the generation, smog chamber exposure, collection, and bioassay of exhaust organics from a light duty diesel vehicle. The simulation of anticipated ambient air conditions was achieved by varying the conditions within the smog chamber. The *Salmonella typhimurium* plate incorporation assay as developed by Dr. Bruce Ames was used to evaluate the particle-bound organics.

MATERIALS AND METHODS

THE SALMONELLA BIOASSAY

The *Salmonella typhimurium* plate incorporation assay described by Ames *et al.* (2) was used to assay the diesel organics. Five histidine-dependent strains (TA98, TA100, TA1537, TA1538, and TA1535) were obtained from Dr. Bruce Ames, University of California, Berkeley. The methods used followed the Ames protocol (2) in view of the suggestions of the Washington conference on the Ames assay (3). Solvent and positive controls done in triplicate were conducted with each test. When possible, the sample was assayed at five doses applied in triplicate. Samples which are reported together were run simultaneously within the cited strain. The mutagenic extracts were tested in the presence and absence of S-9 from Aroclor 1254-induced Charles River CD1 rats. The S-9 fraction was prepared according to the method of Ames (2).

THE SAMPLES

A Mercedes Benz 240D four cylinder diesel automobile run on an engine dynamometer using the Highway Fuel Economy Test driving cycle (HWFET) was used to generate the exhaust exposed to smog chamber conditions. The samples were exposed in the 590 m^3 Calspan Smog Chamber facilities, Ashford, New York. Irradiation was provided by 24 lighting modules arranged in eight vertical chambers. Each array contained two 40-watt sunlamps, eight 85-watt black lamps, and two 215-watt black lamps. The parameters which were varied within the experimental procedure included: (1) presence or absence of UV irradiation, (2) sampling location, (3) length of chamber exposure, and (4) presence of ozone. A summary of the samples is contained in Table 1. Sample 18 was a baseline study in which propylene, SO_2, NO, and NO_2 were injected into the smog chamber without diesel exhaust and irradiated. Samples 20 and 21 were generated within the same experiment. Sample 20 was an immediate sampling of the exhaust particles while sample 21 had a 4 hour dark exposure. Samples 29 and 30 were generated in the same manner as samples 20 and 21; however, sample 30 had a 6 hour UV exposure. Sample 22 was a non-irradiated exposure in which ozone was artifically added to the chamber. The tailpipe sample (24) was collected directly from the automobile tailpipe at near exhaust temperatures. The exhaust gases and particles that passed through the tailpipe filter entered the non-irradiated smog chamber and were collected as sample 23.

After the particles were collected on Pellflex T60A20 glass fiber filters, the filters were folded, sealed in wax paper, placed in a manilla envelope, and stored at refrigerator temperatures. The mutagenic activity of diesel particles stored under these conditions has been shown to be stable (1). Extraction of the particles was with dichloromethane in a soxhlet apparatus. The solvent extract was blown to dryness and solvent exchanged into dimethylsulfoxide.

TABLE 1. EXPERIMENTAL CONDITIONS

Exp. No.	Sample Source:Variable	Sample Number	Exp. Proc.[a]	Irrad.	Gases (ppm)[b]	Prop.[c] Spike (ppmC)	Exposure Time (hrs)
					O_3max		
1	Prop/SO_2/NO_x:Baseline	0018	-	I	*	3.0	6.0
2	Diesel:Baseline	0020	U	No	< .001	-	0.5
	Diesel:Baseline	0021	U	No	< .001	-	6.0
3	Diesel:Baseline	0029	U	I	*	-	0.5
	Diesel:Baseline	0030	U	I	*	-	6.0
4	Diesel:Injected O_3	0022	C4	No	0.65	-	6.0
5	Diesel:Filtered	0023	F	No	*	-	0.5
	Diesel:Tailpipe	0024	T	No	-	-	-
6	Diesel:Tunnel (D-1)	0504	X	No	-	-	-
7	Diesel:Tunnel (D-2)	0505	X	No	-	-	-

[a] Experimental Procedures: U = Emissions injected directly into chamber without filtering or diluting, C = CVS dilution used, F = Emissions filtered at tailpipe and collected from chamber, T = Emissions filtered and collected at tailpipe, X = Laboratory dilution tunnel studies.

[b] Initial concentration only.

[c] Initial propane spike.

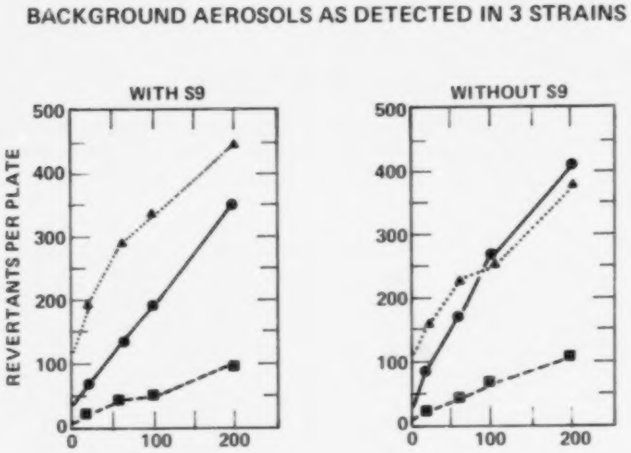
*Equipment failure.

RESULTS

In a baseline study, an artificial mixture of 3.0 ppm propylene, 0.52 ppm SO_2, 0.20 ppm NO, and 0.22 ppm NO_2 were added to the smog chamber without diesel exhaust. Upon irradiation, this mixture generated background aerosols that demonstrated mutagenicity (Figure 1). In order to assess the effect of dark exposure, two samples were collected in the second experiment. The first sample (sample 20) was collected immediately after the diesel exhaust was put into the chamber, and the remaining particles were collected (sample 21) after a 4 hour dark exposure. The third experiment also provided two samples (samples 29 and 30) in the same manner, however, the 4 hour dark exposure was replaced with a 6 hour UV irradiation. Similar mutagenic responses (Figure 2) were obtained from these dark and irradiated exhaust samples collected both immediately and after an extended period.

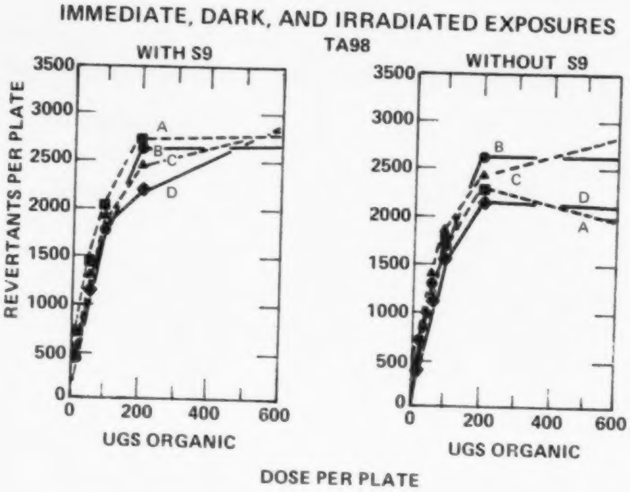
A comparison of the various non-irradiated exposures demonstrates that samples collected at the automobile's tailpipe are significantly different from other non-irradiated samples (Figure 3). Within the linear portion of the dose response curve, the revertants per plate for the tailpipe sample are approximately double that of other non-irradiated samples. Artificially added ozone produced a marked decrease in mutagenic activity of the exhaust organics (Figure 4). Ozone also decreased the bactericidal activity of the exhaust organics. Figure 5 shows a comparison of bioassay results between the smog chamber test vehicle and two dilution samples from a Mercedes 300D automobile. There is a highly significant difference between these two samples. Table 2 provides the mean plate counts at each dose for the reported samples.

Figure 1. Mutagenic response (revertants per plate vs. micrograms of organic extract) of aerosol organics produced upon irradiation of propylene, NO, NO_2, and SO_2.



Footnote: (Δ, TA100; •, TA98; ■, TA1537)

Figure 2. Mutagenicity of irradiated and non-irradiated diesel exhaust organics collected immediately after generation.



(A, Sample 21; B, Sample 29; C, Sample 20; D, Sample 30)

Figure 3. Responses of tail pipe (T-P), immediate (I), and four hour smog chamber (4-HR) exposures of diesel exhaust organic in *Salmonella typhimurium* TA98.

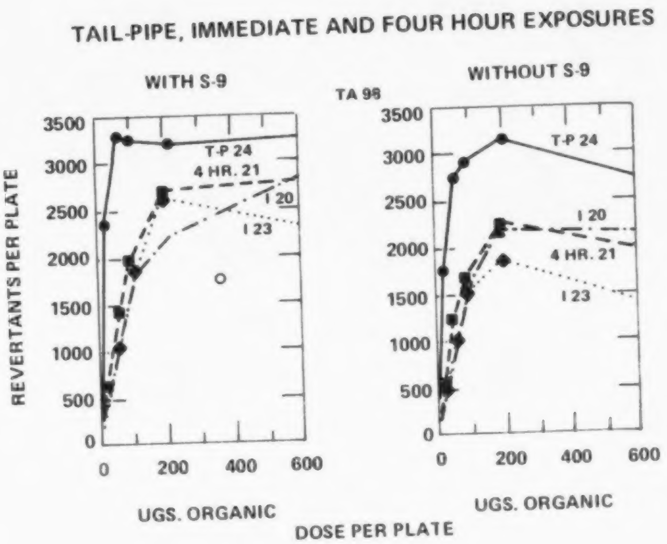
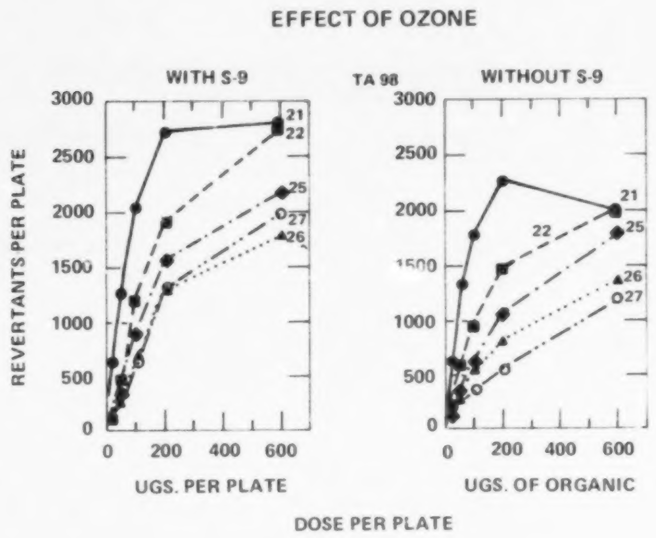
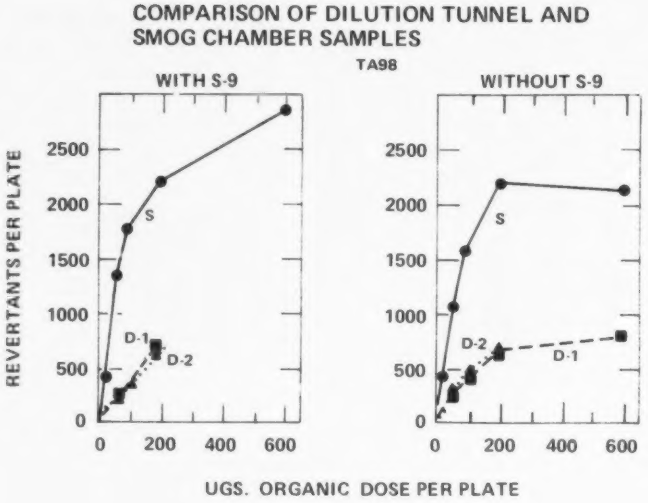


Figure 4. Mutagenicity of diesel exhaust organics exposed in a smog chamber to varying levels of ozone.



Peak levels of ozone: 21, < .001 ppm; 22, 0.65 ppm (injected); 25, 0.012 ppm; 26, 0.24 ppm; 27, 0.54 ppm.

Figure 5. A typical diesel exhaust organic collected from a dilution tunnel compared to a typical smog chamber sample.



S, Smog chamber sample
D-1 and D-2, Dilution tunnel samples

TABLE 2. RESPONSE OF SALMONELLA TYPHIMURIUM TESTER STRAINS TO DIESEL EXHAUST ORGANICS EXPOSED TO SMOG CHAMBER CONDITIONS

			Revertants per plate							
			TA100		TA1537		TA1538		TA98	
Compound	Act	Dose (ugs)	Mean	Std	Mean	Std	Mean	Std	Mean	Std
Positive Control	+	0.50	1388.33	174.26	92.00	14.00	1329.33	42.48	640.67	42.44
	-	3.00	1172.33	28.29	1443.33	39.07	401.33	61.98	259.67	29.02
Negative Control										
Dimethylsulfoxide	+	100.00µl	121.00	28.00	8.67	4.04	36.00	5.57	27.67	2.31
Dimethylsulfoxide	-	100.00µl	102.67	6.43	6.00	1.00	14.33	4.73	20.87	5.03
ARBX-79-0018	+	20.00	189.00	11.31	17.50	0.71	105.50	16.26	63.50	4.95
	+	60.00	285.50	14.85	43.00	1.41	260.00	31.11	130.00	1.41
	+	100.00	332.00	2.83	52.00	1.41	438.50	2.12	187.00	5.66
	+	200.00	443.00	15.56	94.50	23.33	785.50	60.10	350.00	9.90
	-	20.00	155.50	0.71	20.00	5.66	107.50	9.19	82.50	3.54
	-	60.00	227.00	16.97	42.00	1.41	250.50	12.02	171.50	20.51
	-	100.00	254.00	32.53	64.50	2.12	381.00	53.74	261.00	21.21
	-	200.00	381.00	52.33	109.00	1.41	587.00	9.90	409.50	12.02
Positive Control	+	0.50	992.67	63.06	115.33	18.01	907.00	223.43	1914.00	91.15
Negative Control	-	3.00	1273.33	46.50	1133.67	175.16	405.67	11.68	316.00	14.73
Dimethylsulfoxide	+	100.00L	125.33	9.81	16.33	6.66	23.33	2.08	42.00	3.61
Dimethylsulfoxide	-	100.00L	150.00	8.19	12.67	0.58	15.00	7.94	29.00	2.65
Sample 20	+	20.00	428.50	20.51	56.00	7.07	111.00	2.83	407.00	25.46
	+	60.00	802.00	84.85	178.50	7.78	412.50	31.82	1297.00	152.74
	+	100.00	1011.00	12.73	371.50	23.33	626.50	2.12	1800.50	14.85
	+	200.00	1215.50	48.79	702.50	10.61	1144.50	28.99	2198.00	67.88
	+	600.00	960.00	77.78	486.50	4.95	765.00	38.18	2839.00	43.84
	-	20.00	547.00	15.56	48.00	4.24	120.00	46.67	467.50	12.02
	-	60.00	849.50	77.07	160.00	19.80	276.00	43.84	1101.50	21.92
	-	100.00	934.50	31.82	253.00	9.90	402.00	16.97	1599.00	118.79
	-	200.00	834.00	26.87	407.50	9.19	513.50	152.03	2200.00	5.66
	-	600.00	20.50	0.71	***	***	***	***	2130.50	53.03

(continued)

TABLE 2. RESPONSE OF SALMONELLA TYPHIMURIUM TESTER STRAINS TO DIESEL EXHAUST ORGANICS EXPOSED TO SMOG CHAMBER CONDITIONS

			Revertants per plate							
			TA100		TA1537		TA1538		TA98	
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Negative Control										
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ARBX-79-0018	+	20.00	189.00	11.31	17.50	0.71	105.50	16.26	63.50	4.95
	+	60.00	285.50	14.85	43.00	1.41	260.00	31.11	130.00	1.41
	+	100.00	332.00	2.83	52.00	1.41	438.50	2.12	187.00	5.66
	+	200.00	443.00	15.56	94.50	23.33	785.50	60.10	350.00	9.90
	-	20.00	155.50	0.71	20.00	5.66	107.50	9.19	82.50	3.54
	-	60.00	227.00	16.97	42.00	1.41	250.50	12.02	171.50	20.51
	-	100.00	254.00	32.53	64.50	2.12	381.00	53.74	261.00	21.21
	-	200.00	381.00	52.33	109.00	1.41	587.00	9.90	409.50	12.02
Positive Control	+	0.50	992.67	63.06	115.33	18.01	907.00	223.43	1914.00	91.15
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Dimethylsulfoxide	-	100.00L	150.00	8.19	12.67	0.58	15.00	7.94	29.00	2.65
Sample 20	+	20.00	428.50	20.51	56.00	7.07	111.00	2.83	407.00	25.46
	+	60.00	802.00	84.85	178.50	7.78	412.50	31.82	1297.00	152.74
	+	100.00	1011.00	12.73	371.50	23.33	626.50	2.12	1800.50	14.85
	+	200.00	1215.50	48.79	702.50	10.61	1144.50	28.99	2198.00	67.88
	+	600.00	960.00	77.78	486.50	4.95	765.00	38.18	2839.00	43.84
	-	20.00	547.00	15.56	48.00	4.24	120.00	46.67	467.50	12.02
	-	60.00	849.50	77.07	160.00	19.80	276.00	43.84	1101.50	21.92
	-	100.00	934.50	31.82	253.00	9.90	402.00	16.97	1599.00	118.79
	-	200.00	834.00	26.87	407.50	9.19	513.50	152.03	2200.00	5.66
	-	600.00	20.50	0.71	***	***	***	***	2130.50	53.03

(continued)

TABLE 2 (continued)

			Revertants per plate							
			TA100		TA1537		TA1538		TA98	
Compound	Act	Dose (ugs)	Mean	Std	Mean	Std	Mean	Std	Mean	Std
Sample 21	+	20.00	432.50	7.78	66.50	7.78	155.00	11.31	596.50	75.66
	+	60.00	844.50	14.85	222.00	4.24	461.00	16.97	1421.00	106.07
	+	100.00	1070.50	10.61	462.50	43.13	843.00	59.40	2031.00	63.64
	+	200.00	1167.00	33.94	785.00	35.36	1364.00	277.19	2718.50	30.41
	+	600.00	962.00	7.07	392.50	95.46	***	***	2796.00	100.41
	-	20.00	766.00	60.81	74.00	8.49	156.00	25.46	559.50	21.92
	-	60.00	1059.50	75.66	214.00	43.84	413.00	9.90	1341.50	9.19
	-	100.00	1029.50	48.79	335.00	63.64	540.50	12.02	1778.50	43.13
	-	200.00	799.00	55.15	457.50	54.45	389.00	36.77	2280.00	131.52
	-	600.00	4.00	1.41	***	***	***	***	1996.50	103.94
Sample 22	+	20.00	242.00	22.63	24.00	4.24	75.00	12.73	174.50	13.44
	+	60.00	421.50	6.36	80.00	8.49	225.00	26.87	568.50	13.44
	+	100.00	602.50	61.52	154.50	0.71	338.50	0.71	915.00	25.46
	+	200.00	852.00	31.11	277.50	50.20	620.00	59.40	1562.50	65.76
	+	600.00	1143.00	80.61	419.00	33.94	761.00	29.70	2181.50	6.36
	-	20.00	324.50	7.78	41.00	4.24	139.50	6.36	236.00	9.90
	-	60.00	540.50	9.19	92.00	18.38	256.50	36.06	656.50	10.61
	-	100.00	718.50	14.85	141.00	56.57	386.50	70.00	964.50	31.82
	-	200.00	876.00	1.41	272.50	16.26	538.50	40.31	1471.50	68.59
	-	600.00	1020.50	19.09	345.50	17.68	807.50	38.89	2012.50	28.99
Sample 23	+	20.00	346.00	2.83	56.00	7.07	96.00	22.63	310.50	28.99
	+	60.00	728.50	4.95	127.50	20.51	316.00	28.28	1109.50	55.86
	+	100.00	1041.00	8.49	158.50	120.92	657.50	7.78	1702.50	241.12
	+	200.00	1331.00	29.70	640.00	14.14	1028.50	245.37	2628.50	23.33
	+	600.00	909.50	70.00	378.50	31.28	496.50	6.36	2323.50	38.89
	-	20.00	675.50	38.89	47.50	7.78	107.00	14.14	457.00	9.90
	-	60.00	1036.50	36.06	135.50	3.54	296.50	14.85	1011.00	5.66
	-	100.00	875.50	60.10	203.00	42.43	418.00	7.07	1548.00	56.57
	-	200.00	679.50	3.54	263.00	46.67	342.00	74.95	1873.50	140.71
	-	600.00	2.50	2.12	***	***	***	***	1422.50	28.99

(continued)

TABLE 2 (continued)

Compound	Act	Dose (ugs)	Revertants per plate TA100 Mean	 TA100 Std	 TA1537 Mean	 TA1537 Std	 TA1538 Mean	 TA1538 Std	 TA98 Mean	 TA98 Std
Sample 24	+	20.00	866.00	*	425.00	*			2372.00	*
	+	60.00	1020.00	*	1016.00	*			3300.00	*
	+	100.00	779.00	*	603.00	*			3253.00	*
	+	200.00	558.00	*	372.00	*			3210.00	*
	+	600.00	328.00	*	***	***			3248.00	*
	-	20.00	778.00	*	324.00	*			1736.00	*
	-	60.00	1053.00	*	686.00	*			2768.00	*
	-	100.00	805.00	*	589.00	*			2940.00	*
	-	200.00	518.00	*	405.00	*			3169.00	*
	-	600.00	3.09	*	***	***			2750.00	*
Sample 29	+	20.00	308.00	2.83	49.00	8.49			340.00	12.73
	+	60.00	673.00	94.75	136.50	9.19			1044.50	62.93
	+	100.00	912.00	59.40	248.50	23.33			1806.50	51.62
	+	200.00	1124.00	0.00	650.50	28.99			2655.00	43.84
	+	600.00	874.50	43.13	498.00	*			2690.00	49.50
	-	20.00	475.50	27.58	52.50	16.26			411.50	17.68
	-	60.00	861.00	29.70	158.00	12.73			1182.00	48.08
	-	100.00	878.50	51.62	226.00	11.31			1626.50	75.66
	-	200.00	735.50	7.78	338.00	52.33			2054.00	31.11
	-	600.00	***	***	***	***			1790.50	30.41
Sample 30	+	20.00	413.00	7.07	54.50	0.71	120.00	38.18	500.00	50.91
	+	60.00	856.50	33.23	187.50	3.54	412.00	16.97	1404.50	30.41
	+	100.00	1122.00	82.02	341.00	5.66	739.50	3.54	1899.50	3.54
	+	200.00	1397.00	7.07	584.50	50.20	1068.00	152.74	2473.00	128.69
	+	600.00	1121.50	65.76	534.50	20.51	907.50	3.54	2838.00	73.54
	-	20.00	508.00	21.21	77.00	11.31	147.50	26.16	540.50	16.26
	-	60.00	992.00	2.83	220.00	15.56	356.00	80.61	1205.00	24.04
	-	100.00	1055.00	2.83	330.50	21.92	452.00	53.74	1564.00	115.97
	-	200.00	966.00	39.60	430.00	35.36	707.00	32.53	2279.50	6.36
	-	600.00	49.50	33.23	***	***	***	***	2097.00	144.25

DISCUSSION

Previous studies had shown that extracts from diesel exhaust particles are mutagenic when tested in the *Salmonella typhimurium* plate incorporation assay. This study simulated some of the modifying conditions found in an ambient atmosphere containing diesel exhaust. The associated chemistry and engineering procedures are reported more fully elsewhere (4). This study clearly indicates that some mutagenic products are formed by the interaction of a simple hydrocarbon propylene, SO_2, NO, NO_2 gases, and UV irradiation. Earlier studies showed that the spontaneous interaction of simple mixture produces a large variety of organics and five aerosols (5,6). Up to 35% of the total activity of any of the irradiated smog chamber samples could be attributed to this interaction. As shown in Figure 2, the recovery of total mutagenic activity is the same under dark and UV light conditions unless some other mitigating factor, such as ozone, is present. Compared to the remaining non-irradiated smog chamber samples, the tailpipe sample had a marked increase in mutagenicity. This study demonstrates that the response of any exhaust sample, and therefore the substances providing that response, varies markedly with the method of sampling. Various parameters, including collection temperature, concentration of exhaust gases, and rates of chemical interaction, could account for the variation with sampling methods. One factor that was clearly shown to modify the mutagenic activity of particle-bound organics was ozone. As shown in Figure 4, ozone can reduce the mutagenic activity as detected by the Ames test; however, since differing levels of ozone produce differing levels of oxidation and chemical interaction, this study does not demonstrate that all levels of ozone will reduce mutagenic activity. Unfortunately the vehicle used in the smog chamber studies has not, to date, been used in dilution tunnel studies so that direct comparisons can be made between smog chamber and dilution tunnel samples. The closest comparison available is between the smog chamber vehicle and a Mercedes 300D vehicle run with dilution tunnel techniques. The smog chamber sample used for comparison was from a non-irradiated, imediate (0.5 hour) collection. The response between these two samples was clearly different, with the smog chamber sample more mutagenic than the dilution tunnel sample.

In summary, the mutagenic response associated with exhaust particulate organics depends upon the ambient conditions as well as upon the methods of generation and collection. The complex aerosol mixture produced by the interaction of propylene, NO, NO_2, SO_2, and irradiation was shown to be mutagenic. Although UV irradiation without other mitigating factors did not alter the mutagenic activity of the collected organics, ozone was shown to alter the type and level of mutagenic compounds detected by the Ames test. Future experiments will continue exploring the effect of ambient conditions on mobile source emissions.

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General Discussion

J. DAISEY: Did you say how much ozone you put in the chamber?

L. CLAXTON: No, I didn't, but I can tell you; 0.64 parts per million.

R. KLIMISCH: Did you ever get a case where ozone increased the mutagenicity?

L. CLAXTON: No, we have not at this point.

R. KLIMISCH: Why do you say altered; why don't you say reduced?

L. CLAXTON: We haven't tested a variety of levels of ozone. We have only tested one level. I suspect that other things can happen. You may have seen on the slide what appeared as a variety of levels, but those were levels of generated ozone with other mitigating factors, such as different levels of SO_2 and different levels of added reactive hydrocarbon.

P. SCHULTZ: How do you run a blank sample through an Ames Test?

L. CLAXTON: What do you mean by blank sample?

P. SCHULTZ: A sample without the test material.

L. CLAXTON: We always run a series of controls and our controls are always tested with the solvent but without the compound. We have run some compounds that are inert in Ames Tests, as well as in this system.

P. SCHULTZ: Did you have an absolute zero on those?

L. CLAXTON: You always have a level of spontaneous mutation that is recordable.

P. SCHULTZ: Is that substracted from these results?

L. CLAXTON: No, it is not substracted from these results. You can look on the Y axis and see the point of intersection on the Y axis and the level spontaneous.

J. HUISINGH: In the original study that we reported several years ago, the samples that were collected at Southwest Research Institute, blank filters were also run through the total extraction system and were found to essentially contribute nothing to the mutagenic activity. It takes a lot of effort to always run blank samples, and we don't always do that. We have done a number of studies where blank filters were completely extracted and also fuel was extracted and fractionated and we found no activity from those studies.

L. CLAXTON: Probably the best example is actually the fuel sample, which was put on the filters and run through a portion of the fractionation scheme and then through the Ames Test in the same way as the exhaust organics. There was no activity there. It was all at spontaneous levels.

W. THILLY: I thought it was an interesting technical feat to count thousands of colonies on a plate. Are these plates different from ordinary bacterial plates?

L. CLAXTON: The highest count is about 2,000, and we used an automatic colony counter.

W. THILLY: Does the colony counter saturate at around 2,000 counts per plate?

L. CLAXTON: No, but we cannot really go above 2,300 under the conditions in which we are testing colonies per plate. After 2,000 we record too numerous to count.

W. THILLY: Under your conditions of assay, what assurance can you give us that your various samples did not change those numbers of colonies observed as a result of killing the bacteria as opposed to mutating them?

L. CLAXTON: We go back into each of our tests afterwards and take out a number of bacteria that is at least equal and sometimes double to the number of spontaneous bacteria that would occur and we plate each of those colonies out separately to make sure that they are revertents and true mutants.

W. THILLY: That is nonresponsive. Could you answer the question with regard to the number, not their quality of being true mutants.

L. CLAXTON: Are you asking how do you correct for toxicity?

W. THILLY: Quite.

L. CLAXTON: This is a problem in the Ames Test per se in this type of protocol. I realize as you do that you can have killing of almost 95 percent or even more and still have a recoverable number of mutants that is even this high. We have developed, and are working with statisticians to model the results of an Ames Testing and a paper will be published on the subject.

R. YASBIN: We have done the toxicity testing, and there is essentially no more than 90 percent killing with the various extracts that we have gotten from you. Toxicity does not appear to be a problem.

L. CLAXTON: Yes, sometimes when you are testing very toxic compounds, if you get into a high enough level, the number of revertents will drop off in the higher portion of the curve. We generally see very little of that with these diesel samples.

T. BAINES: What was the magnitude of the difference between the tailpipe filter that you took and the results that you normally get from vehicles of that type when you sample them in a dilution tunnel? Would you care to speculate on what you might find when you are able to test that vehicle on a dilution tunnel.

L. CLAXTON: With the filter immediately at the tailpipe sample, it is two to threefold higher for the same type of automobile as compared to most of the dilution tunnels that we have seen. I suspect that if we were able to test the same automobile on a dilution tunnel apparatus, it would be in line with most others.

T. BAINES: What do you think is the difference; what would account for that two to threefold difference?

L. CLAXTON: I don't know. You would need some chemists to help on that.

T. GIBSON: Did you compare diesel particulate exposed to NO_x with diesel particulate exposed to just the other things like SO_2?

L. CLAXTON: To NO_x alone, as compared to the others?

T. GIBSON: Well no, exposed to the mixture, including NO_x, and the mixture for any conditions not including NO_x.

L. CLAXTON: No, generally there is always NO_x from the automobile itself within the mixture.

T. GIBSON: There is no way to do that?

SALMONELLA/MICROSOME MUTAGENICITY ASSAYS OF EXHAUST FROM DIESEL AND GASOLINE POWERED MOTOR VEHICLES

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ABSTRACT

Motor vehicle exhaust from prechamber injection diesel and gasoline powered passenger cars, sampled during US FTP 1973 test cycles and comprising both particulate matter and compounds condensable at ambient temperature, has been assayed for mutagenicity in the Salmonella/microsome test.

Mutagenic components were to a large extent active in the absence of the mammalian microsomal preparation. The mutagenicity of both particulate matter and condensate from diesel exhaust and condensate from gasoline exhaust was decreased in the presence of the microsomal preparation whereas the mutagenicity of particulate matter from gasoline exhaust was enhanced by microsomal activation.

A comparison between the investigated diesel and gasoline exhaust samples shows that the mutagenic effect in the Salmonella test of the diesel exhaust is more than ten times higher than that of the gasoline exhaust.

Fractionation with respect to polarity indicates that the mutagenic components mainly are distributed in neutral aliphatic, aromatic and oxygenated fractions.

Tests for mutagenic monofunctional nitroarenes by an anaerobic assay indicate that such compounds at most are marginally present in the exhaust samples as compared with their presence in airborne particulate matter collected in an urban environment.

INTRODUCTION

Combustion of carbon containing fuels for energy production invariably gives rise to the formation and emission of compounds with potential health effects. In a situation when technological advances as well as administrative and political decisions in some countries have resulted in gross reduction of possible acute and other short-term effects from air pollution, public, political and scientific interests have to some extent focused on long-term consequencies including genotoxic effects, i.e. mutagenic, carcinogenic and allied effects.

An advanced society with foresight may neither wish to allow practices which cause cancer and other somatic genotoxic effects nor want to leave a legacy of an increased mutation rate to future generations. Although combustion emissions are variable and complex, the evaluation of their potential genotoxic effects are not impossible (1).

One of several tools available for the examination of genotoxic effects of combustion emissions is the Salmonella/-microsome test for mutagenicity (2). This screening method is particularly valuable in studies of complex mixtures containing a multitude of compounds not yet available for identification and quantification by chemical analyses. Motor vehicle exhausts which have been assayed for mutagenicity by the Salmonella test in the present investigation are examples of such complex mixtures.

MATERIALS AND METHODS

SAMPLING

Sampling of exhaust gases and conventional exhaust gas analyses were performed by the staff at the Vehicle Exhaust Gas Laboratory of the National Swedish Environment Protection Board. The sampling technique, primarily created for the determination of polycyclic aromatic hydrocarbon (PAH) emission factors, has been described elsewhere (3, 4). The technique is a system in which part of the exhaust gas proportional to the total gas flow is removed and introduced into a sampling train. The sampled exhaust, about 9 % for gasoline and about 5 % for diesel fuel, passes through a cooled glass condenser and the formed aqueous condensate is collected in a glass flask. The cooled gases, at 25 - 50 °C, then passes through a glass fiber filter (Gelman A/E) for the collection of particulate matter. At the end of the sampling period the glass condenser and the flask are washed with acetone and the acetone solution is added to the condensate. All samples were stored dark at refrigerator temperature during the first few days after collection and then at -20 °C until they were processed.

Sampling was performed during complete US FTP 1973 test cycles with warm engines.

Used crankcase oil was obtained either by removing a small amount through the oil dipstick hole immediately after that the engine was turned off or by sampling when the motor oil was changed.

MOTOR VEHICLES

Exploratory studies were performed with exhaust samples from different cars which became available as excess samples in studies (3, 4) of PAH emission factors.

The present investigation concerns two different motor vehicles equipped with standard engines. The gasoline powered car was a Volvo 245L of 1976 which was fueled with reference fuel ERF G1. The diesel powered car was a Peugot 504 of 1978 with prechamber injection which was fueled with commercial diesel oil. Both cars conform with the Swedish exhaust ordinance about CO, hydrocarbons and NO_x for 1976 and later models which is akin to the US federal regulations for 1973 models. In Table 1 are given results of the conventional exhaust analyses expressed as averages of three test cycles.

Table 1. Motor vehicles and exhaust analyses.

Fuel	Diesel	Gasoline
Motor vehicle	Peugot 504	Volvo 245L
US FTP 1973 test, no.	9017 - 19	8905 - 07
Fuel consumption, g / test cycle	820	950
CO, g / test cycle	8.1	117
HC, g / test cycle	4.8	17
NO_x, g NO_2 / test cycle	7.3	17

SAMPLE PREPARATION

All samples were stored frozen, mainly at -20 °C, after they were prepared and between mutagenicity assays.

Particulate matter on filter. Filters were Soxhlet extracted for 16 h with 250 ml acetone. The acetone was first evaporated under vaccuum to about 10 ml and then under a stream of nitrogen on a heating block at < 40 °C to about 0.3 - 0.5 ml. This residue was diluted with dimethyl sulfoxide (DMSO) to a known volume, usually about 4 ml per filter.

Condensate. Apart from that an aliquot of the original solution was assayed for mutagenicity without any further preparations the aqueous condensates were treated in various ways.

A part of the condensate was reduced in volume under vacuum to about 5 % of its original volume and then diluted with an equal volume of DMSO.

A part of the condensate was evacuated mildly until most of the acetone had evaporated. The solution was then passed through a XAD-2 column, the column drained and then eluted with acetone. The extract was reduced in volume and diluted with DMSO as described for filter extracts.

The major part or the whole condensate was extracted three times with half the volume of *n*-pentane. The extracts were combined and reduced in volume and dissolved in DMSO as described for filter extracts with the difference that small amounts of acetone were added before all pentane evaporated. - The aqueous phase from the pentane extraction was also reduced in volume under vacuum and then diluted with an equal volume of DMSO.

Crankcase oil. Fresh and used motor oils were extracted three times with an equal volume of DMSO after which the three extracts were combined.

FRACTIONATION

Fractionation with respect to polarity was performed with a modification of the method described by Wynder and Hoffmann (5). The sample was diluted with diethyl ether and shaken with a equal volume of aqueous 1 M sulfuric acid. The aqueous phase was then removed, neutralized with sodium hydroxide and re-extracted with ether, the ether solution constituting the basic fraction. The first ether solution was further shaken with an equal volume of 2 M sodium hydroxide. The aqueous phase was then removed, neutralized with sulfuric acid and re-extracted with ether, the ether solution constituting the acidic fraction.

The ether solution from which basic and acidic components had been extracted was then reduced in volume and the residue transferred to cyclohexane while residual ether was removed. The cyclohexane sample was applied to a silica gel column which was successively eluted with cyclohexane, benzene and diethyl ether and in some cases also with methanol. The fractions are nominally designated to contain aliphatic (cyclohexane), aromatic (benzene) and oxygenated compounds (ether and methanol).

All fractions were reduced in volume, diluted and dissolved in DMSO as described above.

MUTAGENICITY ASSAY

Mutagenicity was determined with the Salmonella/microsome test with the strains TA 98 and TA 100 using the plate incorporation assay (6). In this test specific Salmonella strains, having histidine auxotrophy, are exposed to the compound(s) on agar plates deficient in histidine. A dose related increase in the reversion frequency above the spontaneous rate to histidine prototrophy is scored as mutagenicity. The exposure is made both in the absence and in the presence of a homogenate from mammalian tissue to simulate mammalian metabolism. The present investigation employed the commonly used microsome containing rat liver supernatant (S-9) from Aroclor 1254 induced male SPD rats supplemented with necessary co-factors as described in ref. 6.

All assays included tests with positive control compounds. The bacterial strains were routinely checked for the presence of known characteristics, spontaneous reversion frequency, sensitivity to ultraviolet light and crystal violet and resistance to ampicillin. Benzo(a)pyrene was used as positive control for the S-9 and 5 µg of this compound has given in the range between 300 and 600 revertants per plate at 20 and 50 µl S-9 per plate in TA 98 in the course of the investigation.

Mutagenicity assays under anaerobic conditions were performed by incubating the agar plates in a BBL GasPak anaerobic system during the first 16 h followed by additional normal incubation for 32 h.

Each sample has been assayed with one plate per dose level in at least three independent test at at least two dose levels. The mutagenic potency, expressed as revertants per liter exhaust, has been calculated from the linear part of the dose response curve by linear regression using each plate as a data point. The standard deviation of the slope of the regression line has also been calculated.

CHEMICALS

The nitroarenes were obtained from the following vendors: 1-nitronaphthalene from Fluka AG, FRG; 2-nitronaphthalene from EGA-Chemie, FRG; 9-nitroanthracene from Aldrich Chemical Co., USA and 3-nitropyrene from Koch-Light, England.

HPLC analyses of the 9-nitroanthracene showed that this sample contains three additional uv-absorbing contaminants. No detectable levels of uv-absorbing contaminants were detected in the other nitroarenes.

RESULTS

Mutagencity assays of extracts of particulate matter and pentane extracts of the condensate showed that mutagenicity was easily detected; see Fig. 1 - 3 and upper part of Table 2. Except for particulate matter from gasoline exhaust all samples gave the highest mutagenic effect in the absence of the S-9 microsomal preparation. The enhanced mutagencity of gasoline particulate matter in the presence of S-9 was, however, only observed with the strain TA 98; this enhancement was maximal at an addition of 20 µl S-9 per plate when the effect of different amounts, 10, 20 and 50 µl, was investigated.

Additional studies with portions of condensates from each fuel showed that extraction with pentane or XAD-2 as well as a simple concentrating result in samples with similar mutagenicity; see lower part of Table 2. The results with the diesel exhaust condensate indicate, however, that none of the preparations recover the mutagenicity completely from the original solution.

FRACTIONATION

Fractionation with respect to polarity of samples of each type of exhaust sample showed clear differences between the two fuels as well as with an acetone extract of urban particulate matter collected by high volume sampling at the roof tops in central Stockholm in March 1979; see Table 3. The recovery of the mutagencity is, however, rather low in the fractionation of some samples.

Table 2. Mutagenicity of extracts of exhaust samples expressed as revertants / liter exhaust ± s.d.

FUEL →		DIESEL				GASOLINE			
TEST CYCLES, NO. →		9016		9017 - 19		8905 - 07		8915 - 16	
STRAIN →		TA 98	TA 100	TA 98	TA 100	TA 98	TA 100	TA 98	TA 100
SAMPLE									
Particulate matter, acetone extracted	- S-9	36 ± 2	72 ± 14	44 ± 3	77 ± 8	1.7 + 0.2	3.8 ± 0.5	4.3 ± 0.5	8 ± 1
	+ S-9[a)]	b)	b)	b)	b)	3.2 ± 0.4	2.0 ± 0.2	7.4 ± 0.5	2.2 ± 0.4
Condensate, pentane extracted	- S-9[b)]	9 ± 2	19 ± 3	17 ± 1	23 ± 1	4.6 ± 0.6	7 ± 1	4.7 ± 0.6	9.5 ± 0.4
aqueous residue	- S-9[b)]	-[c)]	-	5 ± 1	5 ± 1	< 2	< 2	-	-
Condensate, original solution	- S-9[b)]	-	-	32 ± 6	35 ± 9	4 ± 2	-	-	-
Condensate, concentrated	- S-9[b)]	-	-	18 ± 2	20 ± 4	3.7 ± 0.3	4 ± 1	-	-
Condensate, XAD-2 extracted	- S-9[b)]	-	-	15 ± 1		4.8 ± 0.5	-	-	-

a) 20 µl S-9 per plate; b) presence of S-9 decreases the mutagenic effect; c) - not tested.

Table 3. Relative distribution of mutagenicity in different fractions as assayed with the strain TA 98, expressed as per cent of the mutagenicity of the unfractionated sample. Exhaust samples used for fractionation were obtained by combining extracts from several test cycles.

	DIESEL EXHAUST		GASOLINE EXHAUST			URBAN PARTICULATE MATTER	
	9016 - 19		8903 - 07 + 8912 - 16				
FRACTION	particulate matter	condensate[a]	particulate matter		condensate[a]		
	- S-9	- S-9	+ S-9[b]	- S-9	- S-9	+ S-9	- S-9
Basic	2	≤ 4	33	≤ 7	< 2	4	4
Acidic	7	8	4	< 6	5	13	19
Aliphatic	15	6	< 2	< 3	14	< 2	< 2
Aromatic	21	15	32	8	35	15	8
Oxygenated - I	13	3	35	21	10	28	19
Oxygenated - II	-[c]	-	-	-	-	3	4
Sum of fractions	58	32	104	29	64	63	54
Reconstituted sample	-	-	-	-	-	62	60

a) pentane extract; b) 20 µl S-9 per plate; c) - not investigated.

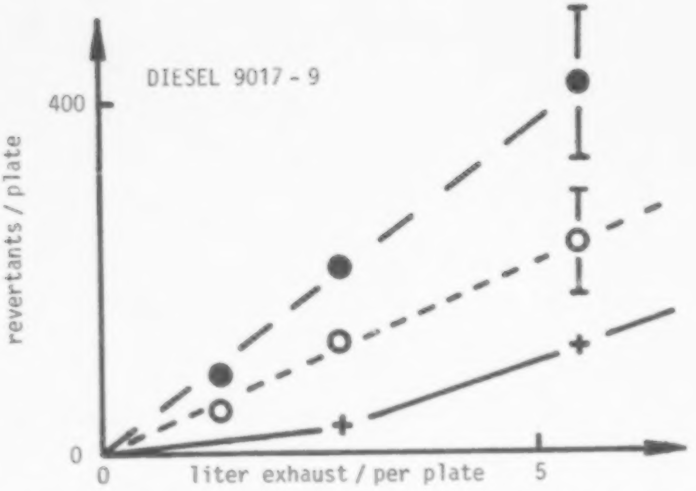

Figure 1. Mutagenicity of the extract of particulate matter from the exhaust of a diesel powered car. Revertants per plate, corrected for the spontaneous reversion frequency, are plotted against liter of exhaust corresponding to the amount of extract per plate. Vertical bars show the standard deviation of some representative points.

● TA 100 - S-9; ○ TA 98 - S-9; + TA 98 + S-9.

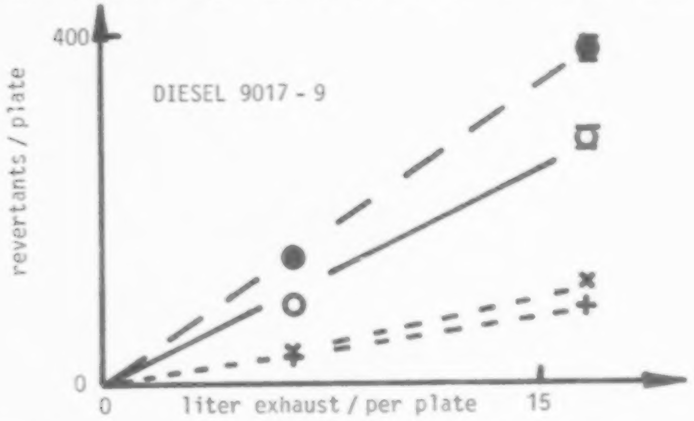

Figure 2. Mutagenicity of the n-pentane extract of the aqueous condensate from the exhaust of a diesel powered car. Cf. figure 1.

● TA 100 - S-9; × TA 100 + S-9;

○ TA 98 - S-9; + TA 98 + S-9.

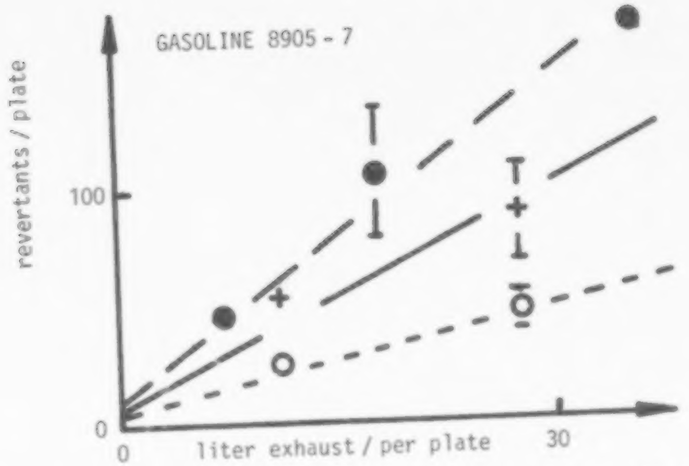

Figure 3. Mutagenicity in TA 98 of extracts of exhaust samples from a gasoline powered car. Cf. figure 1.

○ particulate matter, - S-9
+ particulate matter, + S-9
● n-pentane extract of condensate, - S-9

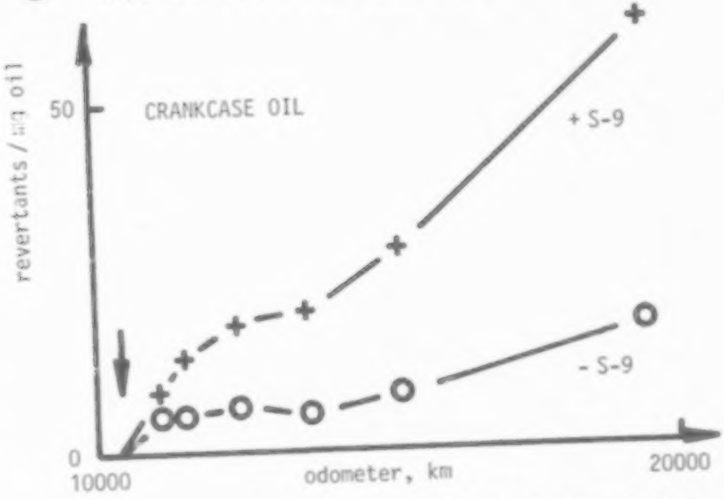

Figure 4. Mutagenicity in TA 98 +/- S-9 of motor oil from a gasoline powered car. Samples were collected at various times between services over a period of 140 days. Fresh, non-mutagenic, < 2 revertants/mg, oil was used from ↓.

NITROARENES AND ANAEROBIC MUTAGENICITY ASSAYS

Some nitro compounds exhibit an enhanced mutagenicity when incubated with some Salmonella tester strains under anaerobic conditions. Typical examples are some monofunctional nitroarenes whose mutagenic responses are given in Table 4.

Table 4. Mutagenicity in the absence of the microsomal system of some nitroarenes in TA 100 and TA 98 under aerobic (normal) and anaerobic conditions. The mutagenicity is given as revertants / µg of the commercial sample assayed without purification.

	TA 100	TA 98	
	aerobic	aerobic	anaerobic
1-nitronaphthalene	15	1.0	2.2
2-nitronaphthalene	24	2.5	5.8
9-nitroanthracene	1	< 1	63 a)
3-nitropyrene	400	3700	8200

a) this increase is caused by the major component of the sample having a retention as expected for nitroanthracene.

Anaerobic incubation of motor vehicle exhaust samples did not cause any considerably increased mutagenicity whereas a significant enhancement has been detected with extracts of urban particulate matter as is shown in Table 5.

Table 5. Relative mutation frequency of exhaust samples and urban particulate matter in TA 100 and in TA 98 under aerobic and anaerobic conditions. The mutagenicity is expressed as per cent of the response in TA 98 -S-9 under normal conditions. The samples are the same as those reported in Table 3.

	TA 100	TA 98	
	aerobic	aerobic	anaerobic
GASOLINE			
particulate matter	200	100	130
condensate	140	100	120
DIESEL			
particulate matter	180	100	130
condensate	150	100	90
URBAN PARTICULATE MATTER	95	100	220

CRANKCASE OIL

The presently employed extraction of mutagenic components from gasoline engine crankcase oil by DMSO has so far given the highest recovery among several tested procedures. Many samples of fresh and used oils have been assayed resulting in no detectable mutagenicity in fresh oils, i.e. < 2 revertants per mg oil, and easily detecable mutagenic effects from used oils, i.e. 5 - 150 revertants per mg oil.

Used crankcase oils contain both compounds which are mutagenic in the absence of microsomal activation as well as compounds which cause an enhanced mutagenicity in the presence of the microsomal system. The mutagenic potency of the motor oil seems to increase with mileage; see Figure 4.

Attempts to assay motor oils from diesel engines have been less successful. Extracts of such oils cause a small increase in the reversion frequency but the effect is not dose dependent which prevents an assessment of their mutagenic potency.

DISCUSSION

Few data exist on the mutagencity of automobile exhausts. Wang et al. (7) give quantitative results with respect to the weight of particulate matter from some gasoline engines and Barth and Blacker (8) indicate that unspecified high mutagenicity of particulate matter from diesel engines, probably direct injection engines, is a reason for concern. Tokiwa et al. (9) have given some data for gasoline exhaust particulate matter and condensate which are very similar to those found in the present study; the same authors also mention that the mutagenicity is less from a catalyst equipped car.

A comparison between the diesel and gasoline exhaust investigated in the present study can be made assigning an average mutagenicity of 100 revertants per liter exhaust for diesel and 15 revertants per liter exhaust for gasoline and using the known fuel consumption and total exhaust volume parameters:

	DIESEL	GASOLINE
Revertants / liter exhaust	100	15
Revertants / g fuel consumed	2 700	190
Revertants / test cycle (12.1 km)	2 200 000	180 000

Subsequent to the present study, Rannug (10) investigated the mutagenicity of diesel and gasoline exhaust samples from passenger cars from the Volvo Co. assaying acetone extracts of particulate matter and diethyl ether extracts of condensates. The diesel exhaust is reported to cause about 3 100 000 revertants per test cycle and the gasoline exhaust between 340 000 - 410 000 revertants per test cycle in TA 100. A catalyst equipped gasoline powered car is further reported to emit much less.

Mutagenicity assays by the Salmonella test have previously revealed the presence in emissions and in urban particulate matter of components which are mutagenic in the test system without requiring the mammalian metabolic activation (2). Similarly behaving compounds are thus also present in the exhaust from diesel and gasoline engines. The exhausts undoubtedly also contain mutagenic/carcinogenic polycyclic aromatic hydrocarbons as benzo(a)pyrene which need mammalian metabolism for the formation of mutagenic metabolites. Their detection in a complex mixture by the Salmonella assay depends on their concentration relative the concentration and behavior of components not requiring mammalian activation. In the present investigation only particulate matter from gasoline exhaust had a composition which favors their detection. Gasoline exhaust has, however, also a considerable S-9 dependent mutagenicity residing in the basic fraction (Table 3).

Techniques used in the sampling of motor vehicle exhaust, stack gases and urban particulate matter can always be discussed with reference to possibilities of artifact reactions during and after sampling. The problems can, however, only be solved with parallel investigations involving different sampling techniques with relevant chemical and biological analyses. - The presence in crankcase oil of mutagenic components not requiring mammalian activation shows that such compounds are formed in the engine and it is reasonable to suggest that they also become a part of the exhaust.

The results of the present investigation underscore the importance of collecting condensable gaseous components as a large part of the mutagenicity is present in the condensates. It seems likely that such components, given sufficient time, may become adsorbed to particulate matter in ambient air.

The fractionation with respect to polarity (Table 3) shows that the distribution of components are dissimilar for the different samples. The wide distribution of mutagenicity among several fractions implies that no single compound or single type of compounds alone are responsible for a major part of the mutagenic effect of any of the samples. - The distribution of the mutagenicity of the urban particulate matter among different fractions (Table 3) is similar to that reported by Teranishi (11).

It has been shown that nitrogen dioxide and nitric acid react with polycyclic aromatic hydrocarbons forming nitroarenes which are mutagenic in the Salmonella system (12). It is, however, not known if such reactions occur in ambient air or on the filter at the time of collection of particulate matter or in both cases.

Some nitro compounds have in the present investigation been shown to have the property of becoming more mutagenic when incubated with the Salmonella strain TA 98 under anaerobic conditions (Table 4). This property has been used to investigate the presence of similarly behaving nitro compounds in environmental samples. Urban particulate matter collected in the Stockholm area shows an enhanced mutagenicity under anaerobic assay conditions as shown in Table 5, but periods with no or little enhancement have also been encountered, probably reflecting differences in the composition of air pollutants. Anaerobic incubation of the diesel and gasoline exhaust samples gave little or no increase in the mutagenicity (Table 4) indicating that mutagenic nitro compounds causing an enhanced mutagenic effect is much less abundant in exhaust samples than in most samples of investigated urban particulate matter.

There undoubtedly exist mutagenic nitro compounds which do not cause an enhanced mutagenicity under anaerobic assay conditions. Several nitro compounds are presently being investigated.

The presence in used crankcase oil of mutagenic compounds which do not require mammalian metabolism has been reported earlier (7) as has the presence of mutagens requiring mammalian metabolic activation (13). The extraction procedure used in the present study may not extract all mutagenic components but it favorably separates mutagenic compounds from components which are toxic to or interfering with the assay. The failure to extract readily detectable amounts of mutagenic components from used motor oils from diesel engines may either be due to that their content of mutagens is low or to that the partition coefficients are unfavorable. Mixing used motor oil from a diesel engine with an equal amount of used motor oil from a gasoline engine results in the extraction of the same mutagenicity as is extracted from the gasoline engine oil alone; this shows that toxic or interfering components from the diesel engine motor oil probably are not causing a false low mutagenicity of the diesel engine motor oil extracts.

ACKNOWLEDGMENT

I am indepted to the staff of the Vehicle Exhaust Gas Laboratory of the National Swedish Environment Protection Board for their kind contribution in supplying well defined samples for this investigation. FK Edward Hefner has contributed with excellently performed Salmonella mutagenicity assays. The investigation has been supported by grants from the National Swedish Environment Protection Board and the Swedish Natural Science Research Council.

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General Discussion

SPEAKER (unidentified): If I understand correctly the main result was that diesel exhaust is ten times as active as automobile exhaust - am I right?

G. LOFROTH: Yes, on the average from this study.

SPEAKER (unidentified): In relation to carcinogenicity, we made a long-term study in Germany with two experts and found just the opposite. Automobile exhaust would be about 40 times as active as diesel exhaust.

G. LOFROTH: I could comment that different biological tests give different results, but regarding the report which you mentioned, I am a little worried about the extraction procedure and preparation. I know what I have been doing with the samples before they went into mutagenicity assays and I was very careful not to destroy compounds.

J. HUISINGH: The condensate that you compared to the filter - is it a condensate that is made after filtration of particles, and how does that compare to the other gentleman's?

G. LOFROTH: No, in this case the condensate was taken before filtration. Of course, one would wish that the first particulate matter filtered would be a condensate. However, from previous studies which have been reported in

Sweden, both with respect to PAH in back gases and also in mutagenicity from the same type of sample, it has been shown that considerable mutagenic activity is present in condensate collected after they have been filtered.

SPEAKER (unidentified): Along those same lines in view of Dr. Bradow's results showing different mutagenicities from different gasoline vehicles, could you describe the particular system of that 1976 gasoline car? I would like to know particularly whether that car had a catalyst, was it run on leaded fuel, and any other exhaust treatment system that may apply to a Swedish car.

G. LOFROTH: This was an ordinary noncatalyst car. There has also been a Japanese report with a few data on a catalyst and noncatalyst equipped car. There was considerable mutagenicity in the noncatalyst car exhaust. Subsequent to the studies we did in Sweden, there had been a larger program in which three Volvo cars, one with catalyst, one without catalyst, and then a diesel car were tested. The same figures came out, the catalyst car had a very low mutagenicity, particularly when the catalyst was warm. In fact the mutagenicity was nondetectable and the ratio between the ordinary gasoline car and the diesel was about the same as in the study reported here, a factor of 10 or a little more.

D. DZIEDSIC: I think a comparison is good. Mouse skin does not give any response to organ-specific carcinogens.

G. EDWARDS: We have built into Salmonella a nitroreductive deficiency, the enzyme that does not require oxygen and have seen no mutagenic reactivity with any of the polycyclic nitrated compounds we have looked at. This would agree with the fact that you get enhanced sensitivity in anaerobic systems implying that the bacteria is making a hydroxylamine or some such intermediate to for a mutagenic metabolite. Secondly, I was interested by something near the end of your talk where you showed that used motor oil showed increased mutagenicity with time of use. Could you give us a couple of details about what kind of motor oil that was; did this require activation; and did you try other bacterial strains, etc?

G. LOFROTH: Mostly the motor oil was from my previous car. I know what type of commerical oil I am buying and putting into my own motor so I could give a few details. The extraction of mutagenicity or mutagenic compounds from used motor oil can be done rather easily by mixing with an equal amount of DMSO and then shaking and separating the phases. Of course one cannot be sure that this is 100 percent extraction, but at least if the concentration increases in the motor oil and there are similar compounds produced in the beginning as well as in the end, one hopes that the extraction is as good in the beginning as in the end. There are compounds which do not need metabolic activation, and then there are additional compounds presumably conventional PAH. It has been shown by chemical analysis that these are present and increasing in used motor oil.

SPEAKER (unidentified): Some years ago there was a report by, I believe, George Gross of Exon Corporation, to the effect that there was observed in a variety of automobiles generation of polynuclear aromatic hydrocarbons in the motor oil. The general hypothesis at that time was the principal hydrocarbons, which are present in motor oil, included cyclo paraffins which were important to the performance of motor oil but could also be converted. I think that would explain the induction with the large effect of S9 in the material you have seen. The reports date, as I recall, to the mid 1960's, so there is evidence in the literature to support the hypothesis that there is some induction of polynuclear aromatics.

BIOLOGICAL AVAILABILITY OF MUTAGENIC CHEMICALS ASSOCIATED WITH DIESEL EXHAUST PARTICLES

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ABSTRACT

To estimate the human health risk of diesel particles it is necessary to know their deposition and retention in the respiratory tract and the rate of dissasociation of mutagenic (and potentially carcinogenic) compounds associated with the particles. The deposition of a chain aggregate aerosol of $^{67}Ga_2O_3$ with size and shape characteristics similar to diesel exhaust particles has been evaluated using the Beagle dog. Approximately one-third of the inhaled activity is deposited in the respiratory tract with most of the particles deposited in lung. The mutagenic activity present in dichlormethane, dog serum, lung lavage fluid, saline, dipalmitoyl lecithin and albumin following incubation of the fluids with diesel exhaust particles was determined in the Ames *Salmonella* system. As observed by other investigators, large quantities of mutagenic activity were removed by the organic solvent, dichloromethane. A very small amount of mutagenic activity was removed by the serum and lavage fluid over a 3 day incubation period. No activity was detected with the other solvents. The minimal mutagenic activity demonstrated in the biological media following incubation with diesel exhaust particles may be due to a lack of removal of mutagens from the particles or an inactivation of removed mutagens by binding or some other process. These preliminary observations will be followed up as an aid in determining the health risks of diesel exhaust particles.

INTRODUCTION

When diesel exhaust particles are extracted with organic solvents, such as CH_2Cl_2, mutagenic activity can be detected in the extract using bacterial test systems (1-2). Since there is a strong correlation between substances which are bacterial mutagens and carcinogens in mammals (3-4) this material must be considered a potential human carcinogen. To understand the magnitude of the risk from exposure to diesel exhaust particles the behavior and fate of both the inhaled particles and their associated organic compounds must be studied. This report describes research directed toward determining (a) the deposition and early retention of ultra-fine particles similar in size and shape to diesel exhaust particles and (b) the biological availability of mutagenic compounds associated with the diesel exhaust particles. The research has demonstrated that a large fraction of the ultra-fine aerosol particles are deposited in the deep lung and a portion are retained for a number of days. Studies with serum and lung lavage fluid suggest that most of the mutagenic activity on diesel exhaust particles is not biologically available either because it is not removed from the particles or because removed mutagens are inactivated by the biological solvent. These approaches provide data to evaluate biological availability of the chemicals which, combined with the data derived from the retention of the particles can be used to estimate the potential chemical dose to lung cells.

METHODS

The method of generating the ultrafine chain aggregate aerosols has been previously described (5). With these same methods $^{67}Ga_2O_3$ aerosols were generated and Beagle dogs exposed by inhalation in a configuration which produced a nose-only exposure. Gallium-67 was selected for use because it has moderate energy gamma-emissions and decays with a half-life of 78 hours thereby providing a useful tag for whole-body counting and gamma camera scanning. This provides a measure of the total fraction of the inhaled material that is deposited, where it is deposited and how long it is retained. Details of the exposure and counting system have been previously published (6).

Diesel particulate materials from two cars were utilized to evaluate the effectectiveness of biological solvents in extracting mutagenic compounds. The samples were selected because, using dichloromethane as a solvent, one had the highest and the other the lowest mutagenic activity of the samples tested to date. The particulate material with the highest activity (Sample 1) was collected on a glass fiber filter, from a 5.7 liter displacement diesel engine, while operating at idle. Sample 2 with the lowest activity was collected on a Pallflex T60A20 filter, from a 2.1 liter displacement engine operating on the Highway FET (Fuel Economy Test) cycle.

Sample 1 was collected at this Institute and Sample 2 was collected at Transportation Systems Center of the Department of Transportation. The percent of total CH_2Cl_2 extractable hydrocarbons was determined for both engines using 1 hour ultrasonic treatment of the particles with CH_2Cl_2. For mutagenesis testing, the CH_2Cl_2 extracted material was evaporated to dryness under nitrogen, diluted in dimethylsulfoxide (DMSO) and tested in Ames *Salmonella* strains TA-98 and TA-100 (7).

Particles were incubated at 37°C for 6, 24, 48, 72 and 120 hours in lavage fluid, serum, saline, albumin, dipalmitoyl lecithin or dichloromethane. The concentrations of the particles in the solutions were 0, 1, 4 and 8 mg/ml for all solvents. Additional tests were conducted using saline, lavage fluid and serum at concentrations of 0, 0.5, 1, 5 and 10 mg/ml. Particles were filtered from the solvents and 0.1 ml added per plate to determine the mutagenic activity in these solvents. This results in concentrations up to 1000 μg of diesel particulate equivalent per test plate. Negative control plates were run for each solvent at each incubation time. Positive controls were run to evaluate the response of the bacteria using sodium azide, 2-nitro-fluorene and 2-amino anthacene. Duplicate plates were used at each concentration. The samples were tested both with and without Aroclor induced liver microsomal enzymes (S-9) for each exposure schedule. Cytotoxicity was determined with a 10^6 dilution of the bacteria plated on histadine supplemented agar. The colonies were counted on an Arteck automatic colony counter after incubation for 48 hours.

The lavage fluid was freshly obtained from a Beagle dog using a previously described technique (8). The cells were removed by centrifugation in an ultracentrifuge at 1000 RPM and the surfactant in the lavage fluid concentrated by centrifugation at 20 000 RPM and resuspended in saline to a final phosphorous concentration of 200 μg/ml. The surface active material in the lavage fluid contains both phospholipids and proteins. DPL was incubated with the particles at 2.8 mg/ml as a representative phospholipid and albumin at 4.6 mg/ml as a representative protein. The serum was also obtained fresh from a Beagle dog and used without dilution. All solutions were filtered through 0.45 μm millipore filters to remove bacteria prior to testing.

RESULTS

Electron photomicrographs and measurements of aerosol properties including count median diameter (CMD), volume median diameter (VMD) and the diffusion equivalent diameter indicate that the diesel aerosol and surrogate aerosol are similar in size, shape and aerodynamic properties (6,9). They are both small chain and cluster aggregates. The inhalation studies (6) indicated that 33% of the inhaled material was deposited in the respiratory tract of the dog. Of the particles deposited, 82% were in the lung and of that in the lung 63% was in the deep aveolar portion of the lung. Twenty-three percent of these particles were cleared rapidly with an effective half-life of less than 1 day. Additional studies are needed to determine the long-term retention pattern, perhaps using an aerosol labeled with a longer lived radionuclide.

CH_2Cl_2 was the solvent which was the most effective in extracting mutagenic activity from diesel particles. The mutagenic response of the extracts of the two particulate samples following 1 hour ultrasonic extraction with CH_2Cl_2 is illustrated in Figure 1. Both TA-98 and TA-100 were used and response was recorded both with and without the liver S-9 microsomal fraction. This characterizes the response of the bacteria to the different exhaust particles extracted in CH_2Cl_2 and serves as a point of reference against which the more biologically relevant solvents can be compared.

The particles were incubated over a number of time intervals in the biological solvents using a range of particle concentrations. Figure 2 shows the mutagenic activity plotted as a function of incubation time for strain TA-100 without S-9. Since the addition of S-9 decreased the magnitude of the response these data are not included in the illustrations on the effectiveness of biological solvents in extracting mutagenic activity from diesel particles. In this figure the bacteria were exposed to extracts of 1000 μg of diesel exhaust particle equivalent per plate. There was little change in level of mutagenic activity in the solvent as a function of incubation time.

The mutagenic activity in the solvents did not change markedly as a function of incubation time, therefore the responses were plotted as a function of concentration using the average of all extraction times. Figures 3 and 4 show the response for both samples tested with strain TA-100 without the addition of S-9. The only biological solvent to show a positive response was serum and it was very slight.

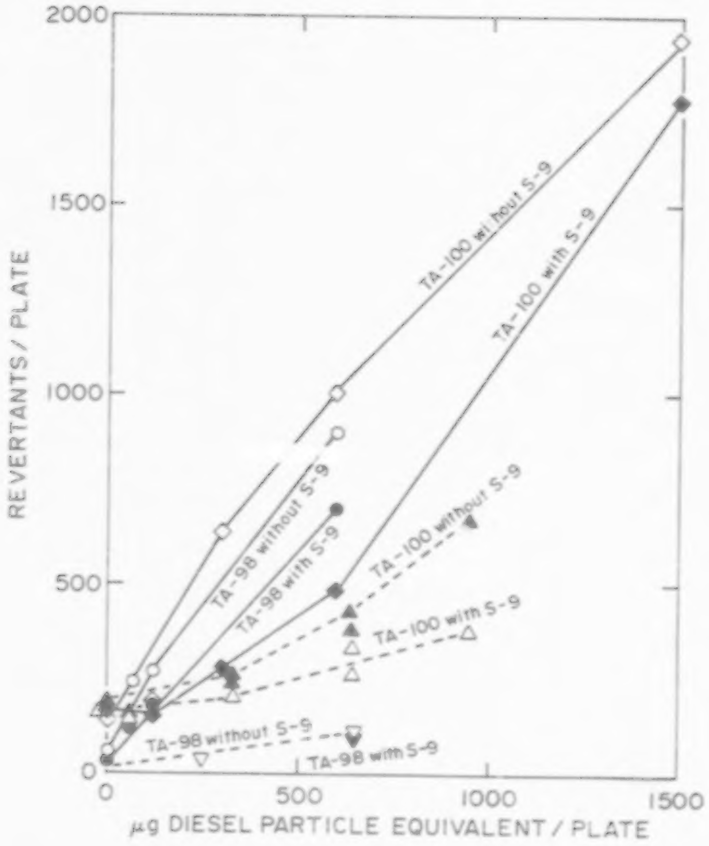

Figure 1. The mutagenic response in TA-98 and TA-100 to CH_3Cl_2 extracts of diesel particle from two different light duty diesel engines.

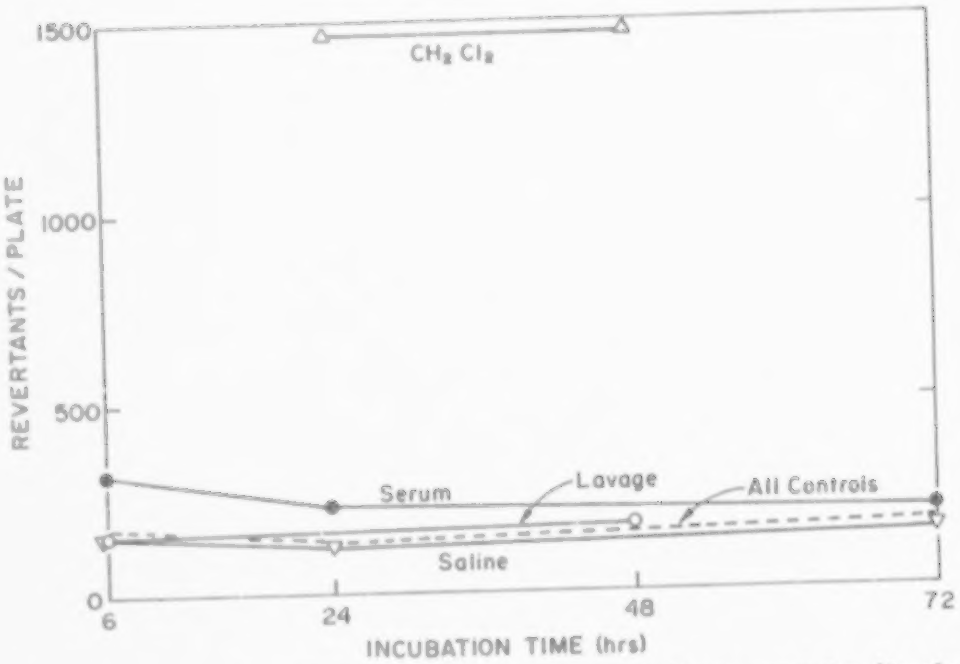

Figure 2. Mutagenic activity extracted from lung of diesel particles (Sample 1) by incubation with a range of solvents as a function of incubation time (TA-100, without S-9).

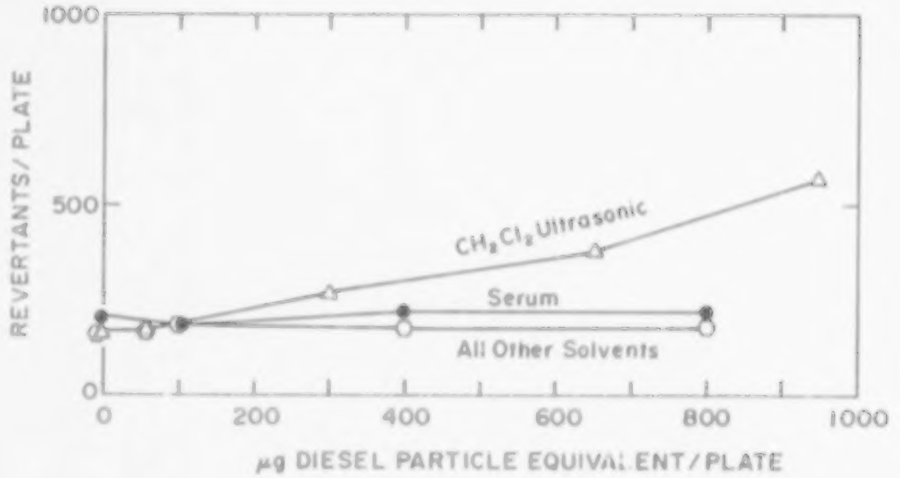

Figure 3. Dose-response relationship for mutations induced in TA-100 by extracts from a diesel particle Sample 2 with a low mutagenic activity.

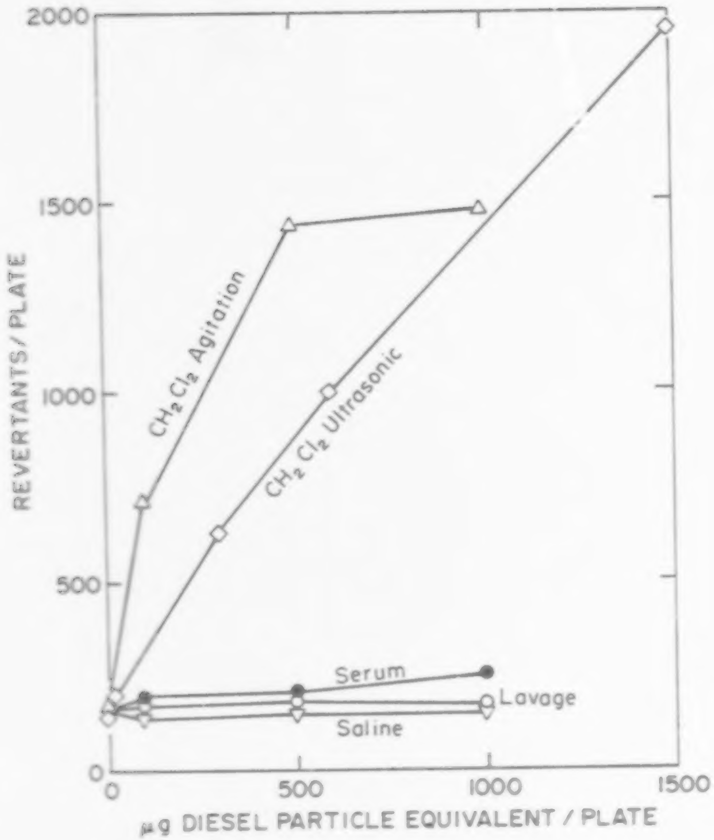

Figure 4. Dose-response relationship for mutations induced in TA-100 by extracts of diesel particle Sample 1 with a high specific mutagenic activity.

When the tests were conducted using bacteria TA-98 on Sample 1 with the high mutagenic activity (Figure 5), there was an apparent dose related increase in mutagenic activity extracted by serum with an indication that lavage fluid could also remove a small fraction of the activity. The slopes of the dose response curves for TA-98 were 1.5, 0.13 and 0.11 revertants/µg diesel particle extracted for the CH_2Cl_2, serum and lavage extraction. Saline extraction failed to show mutagenic activity above that observed in the control cultures.

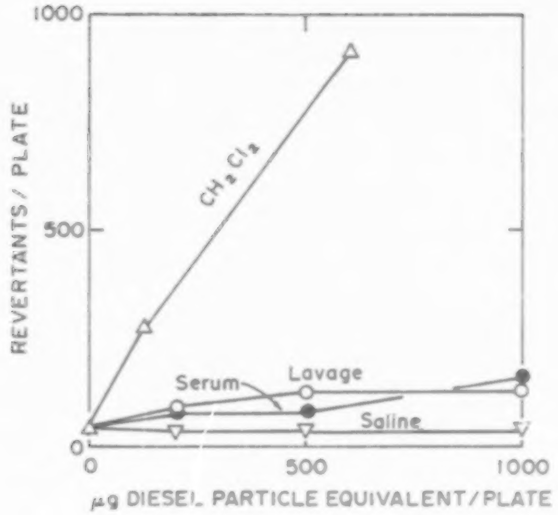

Figure 5. Dose-response relationship for mutations induced in TA-98 by extracts of diesel particle Sample 1 with a high specific mutagenic activity.

DISCUSSION

Small particle aerosols in this study resulted in a higher lung deposition and potential lung dose in Beagle dogs than has previously been reported for aerosols with larger (1.8 µm) particle sizes (10). The deposition in the lung of the dog for the surrogate diesel aerosol was similar but lower than the 88% predicted for man by the model from the Task Group on Lung Dynamics (11). This may be a species difference or may relate to the shape of the surrogate aerosol.

These preliminary results suggest that the deposition and retention pattern for diesel exposures are fairly well predicted by Task Group on Lung Dynamics model (11). The preponderance of the aerosol is deposited in the pulmonary region where clearance is slow. This may have some important toxicological implications since lung retention time of the particles needs to be considered when determining biological availability of the organic compounds associated with the particles.

The estimated chemical dose to lung cells is much more difficult to determine and will require additional research. This question has been addressed by a number of different approaches. One approach was to measure the disappearance of carcinogens from soot in human lungs (12). Another approach involves extraction of exhaust condensates with organic solvents and determining their mutagenic (1-2) and carcinogenic potential (13). In this research an attempt was made to address the question of chemical dose using extraction of mutagenic activity from diesel particles with a range of solvents and determining their activity with the Ames test.

The reversion response of the bacteria from CH_2Cl_2 extracts of the two sources of diesel particles differed by a factor of about 3 for TA-100 and about 10 for TA-98, without S-9. Addition of S-9 did not change the magnitude of the response of TA-98 to extracts from the less active particles (Sample 2). For Sample 1 the addition of S-9 decreased the magnitude of the response for both TA-98 and TA-100. Research on the response of TA-1538 to diesel extracts (2) indicates an enhanced reversion frequency following the addition of liver microsomal S-9 fraction.

When diesel particles were incubated with different media, there was no increase in activity as a function of incubation time over the intervals used in this study. This may indicate that either the mutagenic activity that is soluble is rapidly removed or that an equilibrium is reached between the rate of dissociation of the mutagens from the particles and their rate of inactivation. Thus, the minimal mutagenic response produced by the biological media following incubation with diesel exhaust particles may be due to a lack of removal of mutagens from the particles or the biological materials bind or inactivate the mutagenic compounds after they are removed. The bacteria may not be able to incorporate these bound mutagens and produce a positive response. However, mutagens extracted from fly ash particles by horse serum are capable of producing revertants in the Ames test (14-15). Additional research on this is required to define the movement or binding of chemicals by proteins and surface active substances.

The slopes of the dose response curves can be compared for the serum, lavage fluid and CH_2Cl_2 and indicate that the biological solvents extract from 0 to about 3% of the activity extracted with CH_2Cl_2 as measured by TA-100 without S-9. For TA-98 the mutagenic response for serum and lavage fluid are similar with 10% of the response for CH_2Cl_2 being the maximum. Several potential problems still exist when these results are extrapolated to whole animals. First, they were conducted over a short time and may not reflect the movement of the activity over the long times involved in lung clearance. Second, the solvents themselves, i.e., lavage or serum, may bind or detoxify the mutagenic compounds and make them unavailable for interaction with the bacteria even though they may be removed from the particles and interact with mammalian cells. Finally, the environment in the lungs consists of cells as well as a cellular fluids. The cells may extract, detoxify or activate the mutagenic compounds *in vivo* and make *in vitro* solvent systems less applicable in understanding the changes induced in the whole animal.

These results provide a first estimate of the importance of biological availability of mutagens from diesel particles which can be used in determining the dose to lung cells. Additional research combining *in vitro* inhalation and *in vitro* and *in vivo* testing of mutagenic activity are needed to further define the chemically important dose to lung cells.

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General Discussion

K. CHEN: I have a couple of questions on your deposition experiments. What exactly is the mass medium diameter of the surrogate aerosols, and, specifically, what is the fraction of particles of one micron or below in that surrogate aerosol?

A. BROOKS: I am not an aerosol physicist, but as you heard Dr. Kittleson say there are many ways to measure the size of these particles, and mass medium diameter evidently is not the only or the best way. As nearly as we can tell, the characteristics are very similar to diesel particles. The diffusion diameter that was shown was 0.06 microns, and most of the particles were less than a tenth of a micron. They are chain aggregates, and you can see by looking at them that they are similar. They behave aerodynamically in a similar fashion.

K. CHEN: How did you calculate your deposition efficiency for these particles in adults? Were you able to get the exact activity or did you assume certain respiratory frequency?

A. BROOKS: They know the respiratory volume, the total activity per respiration, and they know the total deposition. From those they can calculate the total fraction deposited as I understand it. They say that 32 percent of the material is deposited.

K. CHEN: You did find about 18 percent in the layrnx and in the trachea?

A. BROOKS: Yes. This is much lower than what you would find with larger particle sizes.

E. CANTRELL: In your studies of the extraction of the biological fluids, I notice that all you had was lipid soluble fluids. It would give the impression that the biological material is not effective in extracting the hydrocarbons, or whatever they are, from the smoke particles. There have been a few recent reports using artificial membranes and human cells to show that lipid material either in the cell membrane or in artificial particles is quite effective in leeching hydrocarbons that are loaded on a particle, asbestos in particular.

A. BROOKS: The mutagenic activity wasn't available for the bacteria. I don't know whether it was taken off or not and tied with the proteins, or whether it was inactivated. We did try to re-extract with that polascitate to try to pull it off the proteins if it was there. We weren't successful. I think that Dr. Huisingh referred to several attempts that she had made to find out where the mutagenic activity has gone. At least we can't get it back from the bacteria. It may be that it is just bound with the protein and that mammalian cells can indeed use it.

J. HUISINGH: Do you shake or sonicate or what do you do during the period that the particles are with the serum?

A. BROOKS: It is on a roller. The particles that are in the serum are rolling in a 37 degree incubator so it is a very mild treatment.

D. KITTLESON: Was the 0.06 microns you gave a number weighted mean diameter?

A. BROOKS: No, that was a diffusion diameter I believe.

D. KITTLESON: Based on number, right?

A. BROOKS: I really don't know the answer to that question. I think there are a lot of people at our place who would really like to talk to you, Dr. Kittleson, about the way that measurement was made and I am sure they could do better than me.

D. KITTLESON: I was just going to say that I was guessing it was a number of mean diameter, because if you look at something like a Volkswagen Rabbit at normal load, the number of mean diameter is around 0.06. The volume or mass weight of mean diameter is then around 0.15, but you have to remember there are different weightings.
That may have confused some of the people when they were asking about what the effects of size were.

DIESEL PARTICULATE MATTER

CHEMICAL AND BIOLOGICAL ASSAYS

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ABSTRACT

This study involves the chemical characterization and biological assay of Diesel particulate matter generated from a single-cylinder, direct-injected Diesel engine. This engine was powered with a pure hydrocarbon fuel. The total extract obtained with this fuel had a positive biological activity by various biological assays, although the raw particulate matter was negative. The methodology of sampling and the assays are discussed together with the implications of the results to date.

INTRODUCTION

Currently there is considerable scientific evidence which suggests that the particulate emissions from a Diesel or a compression-ignition engine have mutagenic or carcinogenic properties. This evidence is based on the results obtained when the organic compounds extracted from the particulate matter are subjected to various bacterial assays. In addition to the biological activity, the physical properties of the particulate matter have also been studied and the average diameters of 95% of the particles were found to be less than μM. Also, these particles have active surface areas which would be capable of adsorbing other combustion products. The combination of this information represents a potential public health hazard since the biologically-active particles are respirable.

A large number of investigators are currently attempting to assess this potential health hazard using various approaches. The approach which has been adopted in this research program is unique since it is attempting to identify the compounds or groups of compounds which have the biological activity and to relate their formation to the fuel and engine operating conditions. The rationale behind this approach is that it is necessary to first identify the origins of the biologically-active compounds before their emissions can be reduced to "safe" levels. A multidisciplinary program, which consists of mechanical engineers, chemists and toxicologists, has been established at The Pennsylvania State University and at The Johns Hopkins University. This group has the necessary expertise to generate and collect Diesel particulate matter and then to characterize it by chemical and biological assays.

This program is attempting to obtain the basic information on the nature of the emissions from Diesel engines by controlling the many interrelated variables. A pure hydrocarbon fuel (2,2,4 trimethylpentane and tetradecane, 50% V/V, cetane number of 53) and a low-ash synthetic lubricating oil were used in place of full-boiling-range Diesel fuels and the traditional lubricating oils. The chemical composition of a full-boiling-range Diesel fuel suffers from inherent variability as a result of the refining processes, the origin of the crude and the aging of the fuel. Also, the chemical composition of the Diesel fuel is unknown since the specifications of Diesel fuels are based on their physical properties. Since the composition of the fuel is unknown and the cylinder and post-cylinder combustion chemistry is unknown, it is difficult to deduce the origin of the biologically-active species. The use of a synthetic low-ash lubricating oil is an attempt to minimize contributions to the emissions from any partially combusted lubricating oil. In addition to the fuel and lubricating oil the Diesel engine was run at a constant set of operating conditions. The rationale behind this careful control of variables was that it should be possible to generate and collect reproducible particulate matter which may be related to the fuel and engine condition. The numbers of compounds in Diesel fuel and in Diesel exhaust emissions are currently thought to be greater than 500 and 10,000 respectively. These figures show the magnitude of the problems facing studies which are attempting to identify and quantify the biologically-active compounds contained in particulate matter from full-boiling-range Diesel fuels.

This report discusses some of the preliminary results from our program in which a light-duty Diesel engine was operated as a chemical reactor under constant conditions. Partial chemical and biological assays have been performed using either the raw Diesel particulate matter or the organic soluble extract. These results are compared to others obtained in a companion study in which an Oldsmobile 350 Diesel engine was run using a refereed full-boiling-range Diesel fuel.

The chemical assays were performed using gas chromatography and/or mass spectrometry and the Ames Salmonella/microsome mutagenicity test[1] and the *B. subtilis* Comp test[2] were used for the biological assays. While the Ames test and the Comp test can never substitute for animal and human epidemiology studies on potential health hazards, they do provide rapid and economical ways of obtaining information about mutagenic and/or carcinogenic activity of a large number of uncharacterized compounds.

EXPERIMENTAL

Engine and Sampling Train

The exhaust particulate matter and gas phase emissions were generated using a small, single-cylinder, four-stroke cycle, air-cooled, direct-injected, Diesel engine (Lycoming Bernard W-51). The raw undiluted exhaust was drawn from the exhaust stream isokinetically. The particulate matter can be collected on either 47 mM or 142 mM membrane filters (Pallflex type T60A20 filter) contained in temperature controlled filter holders. In addition to the collection of the particulate matter, carbon monoxide, carbon dioxide, oxygen, the oxides of nitrogen and the total unburned hydrocarbons were monitored. More detailed descriptions of the engine and sample train have been published previously.[3,4] The lubricating oil (UCON LB-525) was maintained below 70°C with a heat exchanger to reduce thermal decomposition. Before the particulate matter was collected, the engine was allowed to stabilize and the gas phase was monitored before, during, and after the particulate matter was sampled. The gas phase analyses were used to check stability and reproducibility. The typical engine and sampling parameters are shown in Table 1.

Table 1. Engine and Sampling Parameters

Air In-take Temp °C		Air In-take Press. Atm.		Air/Fuel Ratio	Lube Oil Temp °C
42°C		1.00		21	62
Load		RPM		BHP	BSFC
3/4 Rack		2400		3.80	0.600
CO	CO_2	O_2	NO	Total Hydrocarbons	
%	%	%	ppm	ppm	
0.6	9.0	8.5	760	450	

Table 1. continued

Mass of Particulate		Sampling Temp	
(142mM Filter)	(47mM Filter)		
g	g	°C	°C
0.127	0.018	44	500

Chemical Assay

The membrane filters were weighed and then Soxhlet extracted with dichloromethane (approximately 80-100 cycles). The extracts from a series of filters run under identical conditions were combined (5 extracts for the 47mM filters and 2 extracts for the 142mM filters) and the solvent was removed by rotary evaporation. The resulting extract ∿ 4.8% for 47mM filters and ∿ 2.8% for 142mM filters) was weighed and stored in a freezer until used. During extraction, evaporation, storage, and assays exposures to ultra-violet light and plastics were avoided as much as possible.

Before any further steps were taken a gas chromatographic fingerprint was taken using a Micropak column using the conditions shown in Table 2.

Table 2. Gas Chromatographic Conditions for Fingerprint

1.5M, 6mM od, 0.5mM id glass column
3% Dexsil 300 on 120-140 mesh Chromosorb W-HP
Flow Rate 10mL/min.
150°C for 1 min. 150-330°C 16°C/min. Hold at limit 2 min.

If the fingerprint of the extract agreed with previous extracts run under the identical engine conditions within a reasonable error then the extract was submitted for chemical and biological assays. The extract was then analyzed by gas chromatography-electron impact mass spectrometry (Finnigan 4000 GC-MS with INCOS data system). The gas chromatographic conditions are shown in Table 3.

Table 3. Gas Chromatographic Conditions for GC-MS

1.8M, 6mM od, 4mM id, glass column
3% OV-1 on 100-120 mesh Chromosorb W-HP
Flow Rate 30mL/min.
100°C for 3 min. 100-300°C at 10°C/min. Hold at limit 2 min.

Additionally, the extract was analyzed by chemical ionization mass spectrometry using a solids probe inlet (BIOSPECT, Scientific Research Instruments Corporation with a MODCOMP data acquisition system[5]) and by capillary column gas chromatography using the conditions shown in Table 4.

Table 4. Capillary Column Gas Chromatography Conditions

6M., Quadrex glass capillary column coated with Carbowax 20M
Flow Rate 100cm/sec.
50°C for 1 min. 50-150°C at 30°C/min. 150°-210°C at 5°C/min.

Biological Assay

Ames Test

This test system requires the use of genetically constructed mutants of *Salmonella typhimurium*. These strains (TA 1535, TA 1537, TA 1538, TA 100 and TA 98) have been selected for sensitivity and specificity in being reverted from a histidine requirement (auzotrophy), back to non-requiring prototrophy. This mutagenic capacity has been correlated to potential carcinogenic activity.

The following procedure was used to Ames test the total extract. A known mass of extract was dissolved in dimethyl sulfoxide and sterile distilled water was added to give a starting concentration in 50% dimethyl sulfoxide. An aliquot (0.1 mL) of the sample at various concentrations was added to top agar (2.0 mL) containing a 12-16 culture (0.1 mL) of the tester strain. This top agar, cell, and sample to be tested were overlayered onto minimal glucose agar plates and incubated at 37°C for 48 hours. After this time the number of colonies which appeared on the test plates and control plates were counted and recorded. In addition to the extract the raw Diesel particulate matter was also studied. The raw particulate matter suspended in dimethyl sulfoxide was heated gently and then diluted with sterile distilled water (50% soln.) to yield the test solution. When testing with the S-9 microsomal fraction, an aliquot of S-9 (0.5 mL) was added to the top agar containing the bacterial cells and sample, just prior to pouring the overlay.

B. subtilis Comp Test

This test system measures the potential of a chemical to induce or activate DNA repair mechanism. The repair-specific Comp Test detects carcinogens based on the activation of the "SOS" system in clutures of *B. subtilis*. The

"SOS" hypothesis was proposed in order to explain the following phenomena: error-prone repair, induction of prophage, "W" reactivation and "W" mutagenesis, induction of *rec A+* gene product (protein X) and inducible post-replication repair. Transformation experiments necessary for obtaining the results of the Comp test are carried out as described by Yasbin and colleagues.[2] Essentially, cells are grown in GM$_1$ broth, and for 90 minutes (T$_{90}$) after cessation of logarithmic growth (To). The cells are then diluted 10-fold into GM$_2$ broth, and incubated at 37°C on a gyratory shaker for 60 minutes. At this time, DNA extracted from strain RUB818 (1 to 5 μg/mL) is added; the culture is incubated for 20 minutes before the addition of DNAase (10 μg/mL). After an additional five minutes of incubation, the carcinogenic activity of the Diesel particulate is assayed. The transformed cells are incubated with various doses of the Diesel particulate-DMSO solution for 30 minutes at 37°C. After this time the cells are centrifuged, the supernatant discarded, and the cells resuspended in 1X minimal salts. The cells are then plated onto appropriate media for determining the percent transformation. Proper controls for cells not exposed to the test samples are handled in the same manner. The samples for assay were prepared in a similar manner to that used in the Ames test.

RESULTS AND DISCUSSION

Sampling and Chemical Analysis

Considerable effort was expended in order to design and construct the engine and sample train in order to control all the engine and sampling parameters. One hundred and forty data runs were made in order to establish a statistically significant sample. The criteria used to judge the sample generation and collection was the reproducibility of the engine parameters, gas analyses and the gas chromatographic fingerprint of the total extracts. A typical fingerprint of the total extract is shown in Figure 1. The fingerprint is characterized by its shape and the relative peak heights of the various solute peaks. The gas chromatographic column and conditions were optimized for the separation although the efficiency and resolution of the separation are not sufficient for qualitative or quantitative analysis. For comparison purposes the gas chromatographic fingerprint of the total extract from a full-boiling-range Diesel fuel is shown in Figure 2. This particulate sample was obtained from an automobile Diesel engine (350 Oldsmobile) cradled on a dynamometer. The differences between these two fingerprints show graphically the advantage of using the pure hydrocarbon fuel as opposed to the full-boiling-range fuel. The broad unresolved peak in Figure 2 is probably due to the presence of paraffinic hydrocarbons. In all the gas chromatographic studies it was apparent that only 5-10% of the mass of the sample was reaching the detector and was irreversibly adsorbed on the column packing material. This loss in sample had a serious effect when a mass spectrometer was used as a selective detector as it limits the identification of solute peaks.

An interesting observation was made when it was decided to switch from 47 mM filters to 142 mM filters in an attempt to collect larger samples more quickly. The larger filters have a lower pressure drop as a result of filter clogging. When the mass of the total extract from the particulate samples collected on the different sized filters were compared, the particulate matter from the 47 mM filters had a much greater mass extracted than the particulate matter from the 142 mM filters (4.8% *versus* 2.8%). The only difference between these extractions was the size of the Soxhlet apparatus and sufficient data has been collected to ensure that this result is statistically valid. This drastic difference in extraction cannot be easily explained. A possible explanation could be that since the particulate matter is collected in a more dense manner in the 47 mM it acts as a very efficient adsorption surface for any organic molecules in the gas phase. Conversely, the particulate matter on the 142 mM filter is less dense and the gaseous organic molecules have a lower probability of adsorption. Currently we do not have sufficient data to prove or disprove this hypothesis. However, if this theory were correct, it would present a major impact on the study of Diesel exhaust particulate matter because it would mean that it is impossible to collect, by the use of filters, particulate matter which is chemically representative of the particulate matter emitted from the Diesel engine. Additionally, if these volatile organic molecules were the molecules which produced the biological activity, then the potential health impact of Diesel exhaust would have to be totally reassessed. This would mean that the gas phase would have to be collected and studied with chemical and biological assays.

In order to remedy the sample loss on packed columns a preliminary study was performed using capillary column gas chromatography using a splitless injection technique. A gas chromatogram of the total extract from the pure hydrocarbon fuel is shown in Figure 3. Although the coating of the capillary column used for this separation was not the optimum, it was possible to separate this extract into 72 peaks. It is reasonable to expect that with a less polar column and different operating conditions then many of the peaks will be resolved into multi-component peaks. However, it must be stressed that this extract was obtained

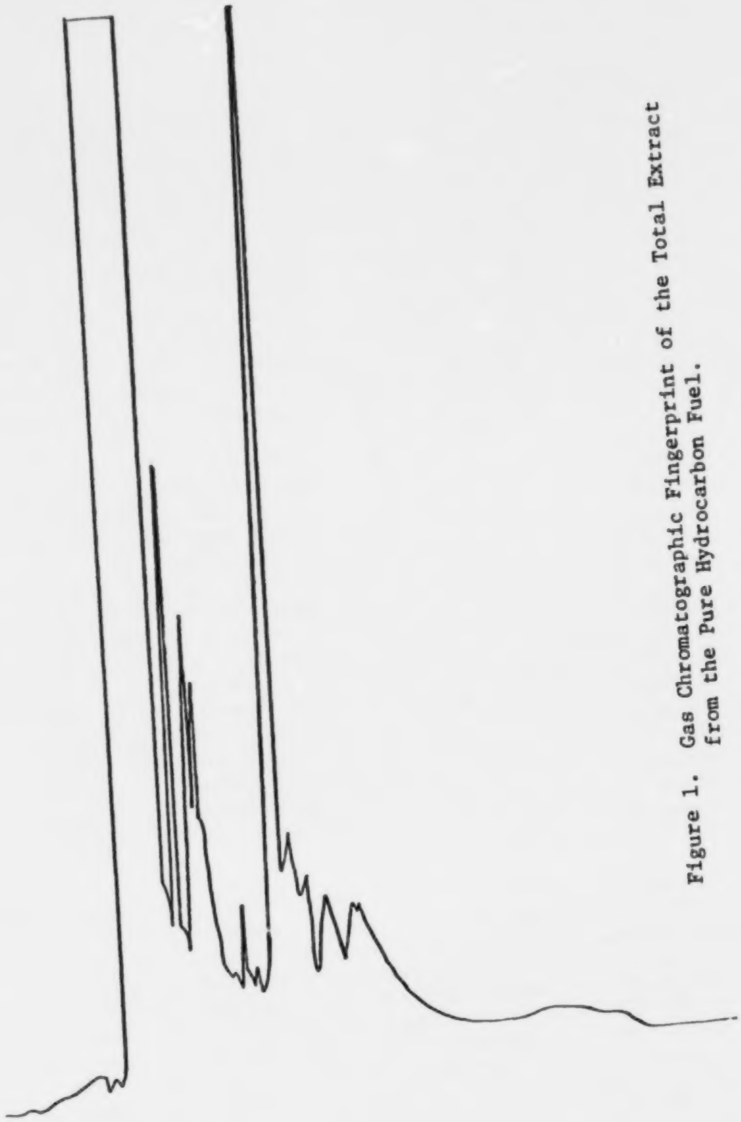

Figure 1. Gas Chromatographic Fingerprint of the Total Extract from the Pure Hydrocarbon Fuel.

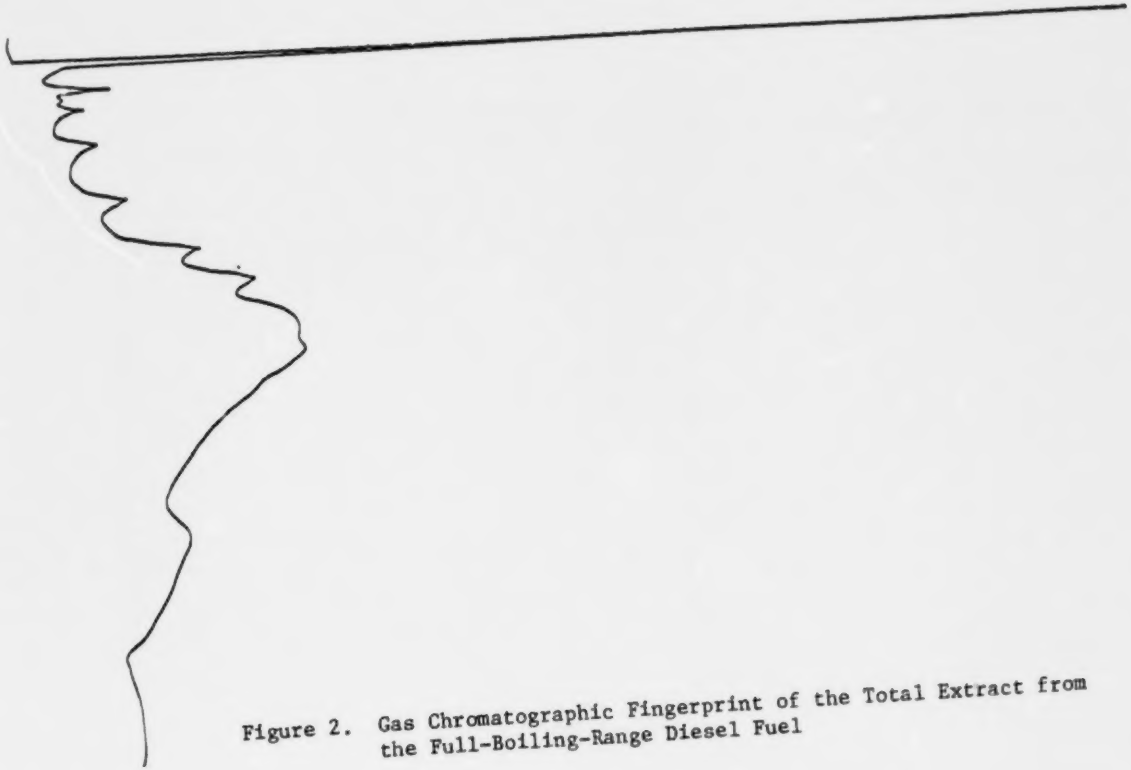

Figure 2. Gas Chromatographic Fingerprint of the Total Extract from the Full-Boiling-Range Diesel Fuel

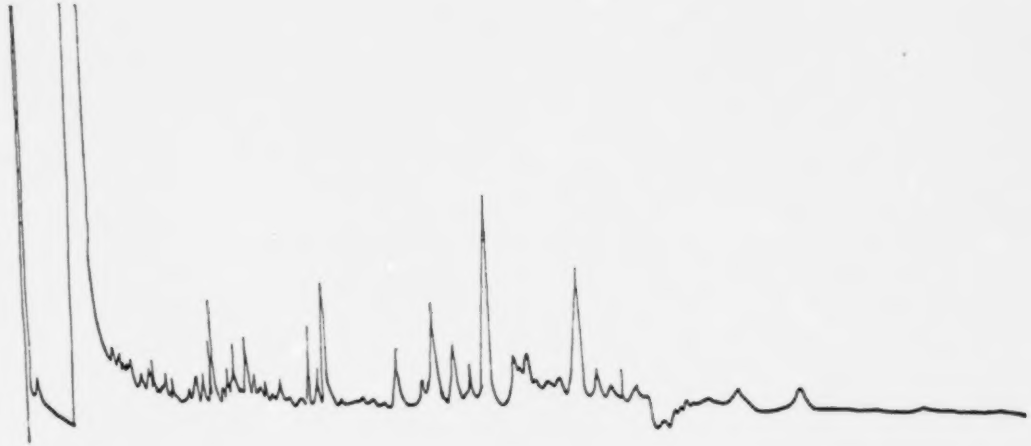

Figure 3. Capillary Column Gas Chromatogram of Total Extract from the Pure Hydrocarbon Fuel.

with the pure hydrocarbon fuel and constant engine operating conditions which shows the wealth of Diesel combustion chemistry. The sample loss through the column was small compared to packed-columns.

Some of the total extracts have been analyzed with gas chromatography-electron impact mass spectrometry. Although the column used for this study had lower efficiency than the Micropak and capillary columns and had significant column bleed, it was possible to make some tentative identifications of compounds contained in the total extract. The gas chromatographic-mass spectrometric data was analyzed using the Biller-Biemann peak searching routine[6] and then the mass spectrum at the peak maximum was compared to the library of mass spectra (INCOS library of NBS mass spectra). Approximately 35 peaks were obtained of which 16 were major peaks but only 7 of these peaks could be identified with significant confidence. The compounds which have been tentatively assigned are shown in Table 5. The plasticizers present in the sample (peak #4) are probably there as contamination which occurs as a result of the contact of dichloromethane with any plastic material. Three total extracts were analyzed and of the 7 peaks which had a tentative assignment 6 appeared in each sample and the relative quantitations of these peaks are shown in Table 6. There is reasonable agreement in these relative quantitations between samples

Table 5. Computer Fit of Mass Spectral Data

Peak #	Fit	Compound
1	970	9H-Fluoren-9-one
	954	Benzo/C/cinnoline
2	922	Phenanthrene
	902	1,1'-(1,2-ethynedyl)bisbenzene
	876	Anthracene
3	932	Benzo/C/cinnoline
	909	9H-Fluoren-9-one
4	980	Butyl-2-methylpropylphthalate
	980	Dibutylphthalate
5	995	1H,3H-naphtho/1,8-CD/pyran-1,3-dione
6	974	Pyrene
	972	Fluoranthene
7	894	Benzo/GHI/fluoranthene

Table 6. Summary of Relative Quantitation

Sample #	Compound	% Total
BS6	Phenanthrene	18.7
BS7	1,1'-(1,2-ethynediyl)bisbenzene	14.3
BS8	Anthracene	18.8
BS6	Benzo/C/cinnoline	4.3
BS7	9H-Fluoren-9-one	2.3
BS8		2.9
BS6	1H,3H-naphtho/1,8-CD/pyran-1,3-dione	21.0
BS7		7.3
BS8		14.4
BS6	Pyrene	26.4
BS7	Fluoranthene	23.5
BS8		21.6
BS6	Benzo/GHI/fluoranthene	2.9
BS7		1.6
BS8		1.9

particularly since the packed column could only partially separate the total extract. The fits of the mass spectra of other solute peaks to the library mass spectra were not significant since it is difficult to deconvolute mass spectra of mixtures of solutes at varying concentrations.

The use of a solids probe with chemical ionization mass spectrometry was disappointing in that no new compounds were identified. This was caused by sample loss during the insertion of the probe and by the plasticizer impurities which overwhelmed the mass spectral data. Currently we are attempting to avoid these problems by the use of a temperature programmed solids probe and by coupling a capillary gas chromatograph to a chemical ionization mass spectrometer. The impurities due to plasticizers have been reduced to a minimum by avoiding any contact with plastic materials.

Bioassay

A statistically significant series of the total extracts from Diesel particulate matter were subjected to both the Ames test and the *B. subtilis* Comp test and the data from these studies are shown in Tables 7, 8 and 9.

The results of these tests, which are used to screen chemical compounds as potential carcinogens, indicate that the total

Table 7. Results of Ames Test done on Total Organic Extract Fraction from Pure Hydrocarbon Fuel (Full* rack, 2400 RPM, A/F 20.0)

	Concentration (μg/plate)							
Strain/Sample	0 (Spontaneous revertants)	3.1	6.3	12.5	25.0	50.0	100.0	50% DMSO (2% per plate)
TA98 BS7	29	39	54	50	88	140	242	21
	31	41	51	40	94	167	261	17
	49	47	65	38	98	171	287	31
TA98 BS9	46	35	34	58	81	115	314	24
	29	27	29	63	83	124	349	14
	37	22	31	49	69	129	330	18
TA98 BS10	40	48	69	90	169	380	650	29
	24	52	78	102	181	397	629	42
	16	41	57	96	146	391	668	50
TA98 BS11	31	41	70	72	135	319	415	33
	37	40	68	78	141	341	443	32
	34	46	56	81	129	311	401	11
TA98 BS12	35	63	76	110	211	448	913	27
	40	69	81	104	156	410	880	49
	46	49	55	121	224	415	962	51

Table 7. continued

	Concentration (μg/plate)							
Strain/Sample	0 (Spontaneous revertants)	3.1	6.3	12.5	25.0	50.0	100.0	50% DMSO (2% per plate)
TA98 BS13	22	$>10^3$	$>10^3$	$>10^3$	$>10^3$	$>10^3$	$>10^3$	31
	28	$>10^3$	$>10^3$	$>10^3$	$>10^3$	$>10^3$	$>10^3$	49
	20	$>10^3$	$>10^3$	$>10^3$	$>10^3$	$>10^3$	$>10^3$	20
TA98 BS14	31	60	91	165	293	488	911	34
	16	49	84	149	260	455	878	29
	11	34	107	170	289	507	940	39
TA98 BS15	28	28	41	82	92	275	432	31
	16	31	52	49	125	216	399	11
	20	36	42	73	140	242	367	37
TA98 BS16	51	58	71	81	131	273	548	53
	47	46	61	110	138	222	497	21
	60	51	49	69	129	251	532	59
TA100 BS16	162	104	120	200	230	514	1036	169
	191	171	110	238	298	556	1174	199
	183	159	108	216	311	571	1003	211

Table 7. continued

Strain/Sample	0 (Spontaneous revertants)	3.1	6.3	12.5	25.0	50.0	100.0	50% DMSO (2% per plate)
	Concentration (µg/plate)							
TA98 BS17	48	79	115	209	440	764	1320	57
	41	81	167	181	382	756	1237	62
	40	92	144	161	412	801	1348	71
TA98 BS18	41	53	74	104	204	360	717	40
	47	59	106	161	259	343	760	31
	46	61	60	159	257	379	692	29
TA100 BS18	161	163	192	168	263	383	563	164
	173	181	197	141	228	361	588	199
	160	164	153	149	241	359	621	232

*Full rack with the pure hydrocarbon fuel corresponds to 3/4 rack with the full-boiling-range Diesel fuel.

Table 8. Results of Ames Test performed with the Total Extract Fraction from Full-Boiling-Range Diesel Fuel. (1/2 Rack, 2000 RPM, A/F ~ 36)

	Concentration (μg/plate)					
Strain	2% DMSO (Spontaneous revertants)	10.0	50.0	100.0	250.0	500.00
TA98	16	28	106	182	397	713
	15	21	92	177	390	882
	14	20	119	184	464	723
	11	34		208	417	824
TA100	288	295	450	540	815	1301
	269	237	346	452	826	1120
	306	286	412	530	849	1490
	264	332	414	511	858	1486

Table 9. Results of *B. subtilis* Comp Test performed with the Total Organic Extract Fraction from Pure Hydrocarbon Fuel (Full Rack, 2400 RPM, A/F ∿ 20.0)

Sample	Maximum Concentration Tested (µg of Sample)	Concentration at which RT = 0.05 (µg/ml)	Survival at which RT = 0.05 %
BS1	200	did not reduce RT to 0.05	93
BS2	200	102	68
BS3	200	did not reduce RT to 0.05	89
BS4	200	164	61
BS5	200	128	13
BS6	200	64	60
BS7	200	172	43
BS8	200	160	98
BS9	200	150	64
BS10	200	did not reduce RT to 0.05	71
BS11	200	did not reduce RT to 0.05	89
BS12	200	140	77
BS13	200	108	68
BS14	200	162	85
BS16	200	110	80
BS17	200	180	62
BS18	200	160	64
Lube Oil	5% (vol/vol)	did not reduce RT to 0.05	100

Table 9. continued

Sample	Maximum Concentration Tested (μg of Sample)	Concentration at which RT = 0.05 (μg/ml)	Survival at which RT = 0.05 %
Tetradecane	20% (vol/vol)	did not reduce RT to 0.05	70
Isooctane (2,2,4 Trimethyl pentane)	20% (vol/vol)	did not reduce RT to 0.05	40 at 1.0% Toxic at $\geq$ 10% to both transformants and viables
Mix 50% tetradecane + 50% isooctane	20% (vol/vol)	did not reduce RT to 0.05	35%
Full-Boiling-Range Diesel Fuel (1/2 Rack,2000 RPM, A/F 36)	500	150	75

extract presents a potential public health hazard. Of the samples which gave negative results in the Comp Test (BS 1, BS 3, BS 10, BS 11) samples BS 1 and BS 3 were later found to have contained large quantities of plasticizers which could cause the extract to precipitate. Samples BS 10 and BS 11 were Comp Test negative although they were Ames Test positive, we do not have a satisfactory explanation for the divergence.

Some preliminary studies have been initiated in which the raw particulate matter has been subjected to the Ames Test and the Comp Test. In addition, the Ames Test was run with the addition of a microsomal S-9 fraction in order to check for the presence of mutagens which require activation. The rationale behind this decision was that it is the raw Diesel particulate matter which is present in the air and not the total extract. Also, we wished to check to see if the biological activity was changed as a result of Soxhlet extraction. Samples of particulate matter which were generated using the pure hydrocarbon fuel and with full-boiling-range Diesel fuels were subjected to these bacterial assays and the data are shown in Tables 10, 11 and 12. The particulate samples from the full-boiling-range Diesel fuels were collected under different load conditions.

Table 10. Results of Ames Test Raw Diesel Particulate from a Pure Hydrocarbon Fuel.

Raw Particulate RPM 2400, Full Rack AF∿20 TA 98	Concentration (µg/plate)			
	0	197	485	970
	(Spontaneous revertants)			
	61	41	50	49
	64	32	47	53
	39	36	32	35
	21	34	30	36
	With S-9 Activation			
	83	51	62	56
	89	40	74	61
	41	48	44	51
	47	42	37	66

Raw Particulate RPM 2400, Full Rack AF ∿ 20 TA 100	Concentration (µg/plate)			
	199	183	167	194
	231	187	178	211
	121	120	118	127
	116	133	111	131

Table 10. continued

TA 100	Concentration (μg/plate) 0	197	485	970
With S-9 Activation				
	212	211	184	196
	214	200	199	162
	140	143	134	161
	137	150	143	135

The raw diesel particulate was soluble in 50 % DMSO.

Table 11. Results of Ames Test with Raw Diesel Particulate from a Full-Boiling-Range Diesel Fuel.

Raw Particulate
1/2 Rack, 2000 RPM
AF∿36

	Concentration (μg/plate) 0 (Spontaneous revertants)	56	112	224	548	1096
TA 98						
	50	59	64	58	71	64
	41	74	58	51	63	52
	41					
	48					
With S-9 Activation						
	60	61	51	63	75	61
	53	49	59	68	82	51
	58					
	51					
TA 100	187	184	181	181	211	196
	180	167	297	183	223	201
	172					
	169					
With S-9 Activation						
	197	201	187	200	231	218
	190	179	199	181	211	213
	178					
	210					

Table 11. continued

Raw Particulate
3/4 Rack, 2000 RPM
AF∿25

Concentration (μg/plate)

TA98

0 (Spontaneous revertants)	167	333	666	1332
42	40	41	46	68
51	41	49	41	52
47		38	49	
41		60	43	
With S-9 Activation				
58	61	47	54	63
60	83	32	42	67
59		58	53	
49		56	58	
TA 100				
171	165	198	201	197
191	181	204	224	211
With S-9 Activation				
193	200	189	218	193
198	169	222	189	167
163	189	162	183	
191	197	195		

Raw Particulate
Full Rack, 2000 RPM
AF∿20

Concentration (μg/plate)

TA 98

0 (Spontaneous revertants)	125	250	500
44	51	70	74
57	48	64	61
63	37	56	83
41	31	60	68

Table 11. continued

Concentration (μg/plate)

TA 98

0	125	250	500
With S-9 Activation			
61	62	73	81
58	49	83	59
53	53	75	47
51	59	89	84

Raw Particulate
Full Rack, 2000 RPM
AF 20

Concentration (μg/plate)

TA 100			
171	187	211	210
192	183	201	190
201	169	203	187
200	191	208	222
With S-9 Activation			
201	193	221	208
205	210	215	193
223	216	200	186
196	211	195	219

Raw Diesel particulate samples from the Oldsmobile Diesel engine were not soluble in DMSO. The mass of sample to be tested was placed in DMSO, heated gently, and sterile distilled H_2O was added to yield a 50% DMSO solution.

Table 12. Results of B. subtilis Comp Test on Raw Diesel Particulate.

Sample and Condition	Maximum Concentration Testes (μg of sample)	Concentration at which RT = 0.05 (μg/mg)	Survival at which RT = 0.05
Pure Hydrocarbon Fuel RPM 2400 Rack Full A/F 20.76	776.2	did not reduce	71%
Full-Boiling-Range Diesel Fuel 1/2 Rack, 2000 RPM A/F 37	1240	1,100	90%
1/2 Rack, 2000 RPM A/F 37	1420	1,320	↑100%
Full Rack, 2000 RPM A/F 20	6200	did not reduce	28%
Full Rack, 2000 RPM A/F 20	6200	did not reduce	35%
3/4 Rack, 2000 RPM A/F 25	6200	did not reduce	47%
3/4 Rack, 2000 RPM A/F 25	6200	did not reduce	51%

All these samples proved to be Ames Test negative with or without microsomal fraction. Only one sample (Full-boiling-range Diesel fuel 1/2 rack) appeared to give positive results in the Comp Test, however we do not have sufficient results to know whether this result is significant.

CONCLUSIONS

This model approach to Diesel engine exhaust emissions has shown that simple hydrocarbon fuels produce exhaust particulate matter which has biological activity. This suggests that it is the reaction mechanisms found to occur under engine combustion conditions which account for this biological activity. However, although the biological activities were observed for the total extract the raw particulate matter did not exhibit similar properties. This anomaly may be very significant since it could mean that the compounds adsorbed or chemisorbed onto the particles are not readily solubilized under physiological conditions and therefore do not represent a serious health hazard. However, since the particles are smaller than 1 μM in diameter they can be deposited in the alveoli and the clearance time could be sufficiently long to allow the compound to become bioavailable via the macrophage. An alternative explanation is that it is purely a concentration effect since the total extract only represents 4-8% of the mass of the raw particulate matter. Currently studies are in progress to measure the heats of adsorption of various compounds onto particulate matter in an attempt to estimate the magnitude of the adsorption energies. Also the size, shape, surface area and porosity are under investigation. No attempt has been made to confirm the preliminary identification of the compounds found in the total extract nor have their biological activities been measured, although this work is in progress. The effect of filter size on the percent extraction is very interesting and could be very significant since if the use of filters to collect particulate matter does produce particulate matter enriched with organic compounds, then the biological activity of Diesel particulate matter may be overestimated.

Another aspect of the use of filters to collect particulate matter is that the particulate matter may be subject to chemical reactions as a result of volatile reactive species. An excellent recent publication by Pitts[7] has shown that benzo(a)pyrene reacts with nitrogen dioxide to produce mononitrobenzo(a)pyrene which is mutagenic without activation. It is reasonable to expect that other compounds could undergo similar reactions with the oxides of nitrogen which are present in Diesel exhaust. This hypothesis may also be significant when the overall public health assessment is made. It is interesting to note that the bacterial activities of the extracts from particulate matter of the pure hydrocarbon fuel and the full-boiling-range fuel do not differ significantly. These results suggest that the bacterial activities which have been observed for extracts from particulate matter from Diesel engines are the result of combustion and are not artifacts from the fuel. It is expected that the methodology discussed herein will provide chemical identification of the biologically active compounds contained in Diesel exhaust emissions.

In future studies we will expand our studies with raw particulate matter to include animal and macrophage tester systems. Also, we plan to vary the engine operating conditions and blend additional fuels with our pure hydrocarbon fuel.

ACKNOWLEDGEMENTS

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General Discussion

J. DAISEY: Could you tell us how you determined your loss of material on your GC column, the dexal column versus the capillary column?

T. RISBY: I made an estimate in terms of math and in terms of response of the flamminization detector versus the amount injected. When I was making the injections, it seemed that the response - that is, the amounts of signals that I should get versus the amount that I was getting, was very much lower.

J. DAISEY: Were the amounts injected the same for both these types of columns?

T. RISBY: For the count, yes. We were injecting one microliter of the same concentrations and we were using split injections.

J. HUISINGH: I don't think it is proper to say you are testing the particle alone when you suspend it in dimethyl sulfoxide, because it has been clearly shown by a number of different people that the DMSO is also extracting some materials.

DIESEL PARTICULATE EXTRACTS IN CULTURED MAMMALIAN CELLS

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ABSTRACT

It has been reported that particulate material from diesel engine exhaust contains bacterial mutagens. To determine whether mammalian cells are sensitive to these or other mutagenic substances, extracts of the diesel particulates (DP) have been tested in the Chinese hamster V79 cell line by our laboratory and in the mouse lymphoma cell line L5178Y by Litton Bionetics. Two dichloromethane extracts of DP were tested for cytotoxicity and mutagenic activity at concentrations up to 350 μg of extracted material per mL culture medium.

Diesel particulates were collected from the exhaust of a General Motors diesel engine at 100°C using Pallflex filters or an electrostatic precipitator. Extracts were produced by Soxhlet extraction with dichloromethane for 4 hours. Treatment of the V79 cells with up to 100 μg diesel extract per ml of culture medium for 5 to 16 hours decreased cell survival substantially compared to control cells, but resulted in no significant changes in their mutation frequency. Addition of a rat liver enzyme preparation reduced the relative toxicity of the diesel extract with no increase in the mutation frequency. Concentrations of 200-350 μg/mL were active in the mouse lymphoma cell assay, although the extract was also very toxic at these levels.

INTRODUCTION

Concern about the health effects of diesel exhaust particulates has centered on their potential action as carcinogenic agents. The bacterial assay system (Ames test) has detected mutagens associated with the diesel particulate. Although valuable as a preliminary screening test, this assay may not reliably indicate the relative potency of chemicals for mammalian cells because of their more complex cell structure and/or different metabolic pathways. The mutagenic activity in an extract of diesel particulates may also vary, depending on the type of engine and collection and extraction methods [1,2].

We have tested extracts of diesel particulate from a GM 5.7 L engine produced by Oldsmobile for mutagenic activity in Chinese hamster V79 cells. In addition, one sample was tested under contract by Litton Bionetics using the mouse lymphoma L5178Y cell assay system described by Clive and Spector [3]. These extracts were all mutagenic to the bacterial strain *Salmonella typhimurium* TA98 [4].

The V79 cells were one of the first cell lines used for the demonstration of the chemical induction of mutations in mammalian cells [5]. The permanent alterations in the cellular DNA are translated into specific RNA and protein molecules and can be detected with selective conditions which allow the mutated cell to grow where normal cells die. The V79 cells have been used here with the selective agents ouabain, which is thought to select for cells with an altered NA^+,K^+ATPase, and 6-thioguanine and 8-azaguanine, which select for cells deficient in the enzyme hypoxathine-guanosine phosphoribosyl transferase (HPRT). These selection procedures are the ones used most frequently with the V79 cell line. Because only one functional HPRT gene appears to be present in the cells, a larger number of mutants can be recovered than if two genes must be inactivated before the cells can be selected. Although the Na^+, K^+,ATPase gene may be present in more than one copy, the selection process is feasible because ouabain selects for a "dominant" mutation; only one gene must produce an enzyme not inhibited by ouabain for the cells to survive. In the mouse lymphoma cell assay, the thymidine analogues 5-bromo-2' deoxyuridine or 5-trifluorothymidine select for cells which have lost their thymidine kinase activity. The L5178Y cells in this assay have been previously selected so that they contain only one active thymidine kinase gene.

METHODS

The Chinese Hamster V79 cell line was initially obtained from Dr. E. H. Y. Chu of the University of Michigan. The cells grew as a monolayer in plastic dishes containing Dulbecco's Modified Eagle's medium (DME) supplemented with 5% heat-inactivated fetal calf serum. They were incubated at 37°C in 10% CO_2 and routinely subcultured twice a week after treatment with 0.05% trypsin to dissociate them from the dish. The cell number in the trypsinized cell suspensions was determined using an electronic particle counter.

Diesel particulates were generated by a General Motors 5.7 L diesel engine produced by Oldsmobile. Two different samples of particulates were extracted for testing with V79 cells. The first sample, DP(1), was collected on a Pallflex filter at 100°C exhaust temperature from an engine burning Marathon #2 fuel. The second sample, DP(2), was collected at 100°C on an electrostatic precipitator from an engine burning Amoco 2D federal compliance fuel. Both particulate samples were extracted with dichloromethane on a Soxhlet apparatus for 4 hours. The solvent was then evaporated and the residue weighed to calculate the percent extractable material. DP(1) and DP(2) yielded 4.7 and 8.9% extractable material, respectively, labeled DPE(1) and DPE(2). The particulate sample collected for testing in the mouse lymphoma cell assay was similar to DP(2), except it contained 11.6% extractable material. The collection and extraction conditions are described by Chan et al [4].

The diesel extracts were dispersed in dimethyl sulfoxide (DMSO) to make stock solutions of up to 35 mg/ml. This was well mixed and diluted in culture medium immediately prior to cell exposure. The final concentration to which the cells are exposed is referred to as the amount of diesel extract per mL of culture medium. A typical concentration, 100 μg/mL, is 100 μg of extractable material from diesel particles per mL of culture medium. The total amount of extract in the exposure period is the concentration (μg/mL) times the volume of medium on the cells (5 mL/25 cm^2 flask, 10 mL/75 cm^2 flask). The amount of diesel particles which would give an equivalent exposure level is dependent on their extractablility; for example, cells in 10 mL of culture medium with 100 μg/ml of DPE(2) are exposed to substances from a total of 11 mg of whole particles.

For activation in the V79 cell system, a microsomal enzyme preparation (S9) was prepared as described in [6] from rats induced with 3-methylcholanthrene. S9 (8-20 μL/mL culture medium) was added in some experiments to investigate the effect of enzyme catalyzed alterations of the diesel extract. Cofactors glucose-6-phosphate (0.15 mg/mL) and NADP (0.3 mg/mL) were added with the S9 as specified. The S9 used by Litton Bionetics was prepared from Aroclor-induced rats; 50 μl/ml was used in the cell suspension to study activation.

Cytotoxicity

Survival of the V79 cells in each experiment was investigated by measuring their relative cloning efficiency. During the first subculture of cells after the exposure period, 400 cells are plated separately in small culture dishes (60 mm dia., 100 cells/dish). Each viable cell will attach and divide many times (one cell division per 12-15 hours) to form a colony about 1-2 mm in diameter within a week. These colonies are stained and counted. One hundred untreated cells will normally form 70-80 colonies (based on ∿15 experiments), giving a cloning efficiency of 70-80%.

In a specific experiment to measure the cytotoxicity of DPE(2), cells were plated in 12 small dishes (4×10^5 cells/ 35 mm dia. dish) and exposed for 16 hours to concentrations of DPE(2) from 0 to 100 μg/mL. After exposure, the cells were trypsinized and 400 cells from each dish were plated and cultured as described above.

In the mouse lymphoma assay system, cytotoxicity is measured as the reduction in growth of the cells in suspension compared to the growth of untreated cells. Their plating efficiency is measured only at the end of the expression period for calculation of the number of viable cells treated with the selection agent.

Mutagenicity

The mutagenicity assay conditions are listed in Table 1. V79 cells were plated in 25 or 75 cm^2 culture flasks (1-4×10^6 cells/flask, 1 flask/test condition) and incubated at least 5 hours before exposure to test chemicals. The substances to be tested were first dissolved or diluted in DMSO to make a solution 100 times the final concentration. N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) and benzo[a]-pyrene (B[a]P) were used as positive controls for direct-acting and indirect-acting mutagens, respectively. To start the exposure period, the solutions of chemicals in DMSO were added directly to a flask containing cells and a known volume of culture medium. For investigation of enzymatic activation, S9 and its cofactors were mixed with the culture medium prior to its addition to the culture flask.

TABLE 1 MUTAGENICITY ASSAY CONDITIONS

Cell Line/Selection Agent	Exposure Period	Expression Period	Selection Period
Chinese Hamster V79/ouabain	16 hours	2 days	7 days
Chinese Hamster V79/ 6-thioguanine or 8-azaguanine	5-16 hours	6-11 days	7 days
Mouse Lymphoma L5178Y/ 5-bromo-2'-deoxyuridine or 5-trifluorothymidine	4 hours	2 days	10 days

At the end of the exposure period, the cells were washed with two changes of normal medium. Cells in each flask were trypsinized, counted and replated. For selection with ouabain, 10^6 cells or more were plated in 100 mm diameter dishes (1-2x10^5 cells/dish). Medium containing 1 mM ouabain was added 2 days later.

For selection with 6-thioguanine (6-TG) or 8-azaguanine (8-AG), an expression period of at least 6 days is required for optimum recovery of the mutant cells. During this time, the cells were subcultured 2 or more times because of their rapid growth rate. At the final subculture, 5x10^5 cells or more were plated (0.5-1x10^5 cells/100 mm dish). The selection agent was added after 1-3 days. Concentrations of 30 μg/mL 8-AG and 4 μg/mL 6-TG were used. The culture medium with 6-TG consisted of DME and 5% dialyzed fetal calf serum; undialyzed serum is reported to contain substances which interfere with 6-TG uptake in the cells.

Cells were incubated with the selection agents at least 7 days, then were fixed with 100% ethanol and stained with Giemsa. Colonies of more than ∿50 cells were counted.

The mouse lymphoma assay was performed by Litton Bionetics as described by Clive and Spector [3]. Briefly, the cell suspensions were treated for 4 hours with the test materials. Ethylmethane sulfonate (0.5 μL/ml) was used as a direct-acting positive control; dimethylnitrosamine (0.3 μL/ml) as a positive control for activation of mutagens by S9. After two days the cells were plated in agar (0.35%) with the selection agents 5-bromo-2'-deoxyuridine (100 μg/ml) or 5-trifluorothymidine (3 μg/ml).

RESULTS

Cytotoxicity

The cellular toxicity of the diesel extracts (DPE) was an important factor in these studies as detection of small increases in the mutation frequency requires that a substantial number of cells (10^5 or more) must remain viable after the treatment. This survival factor must be balanced against the fact that the mutation frequency (number of mutants/number of suriving cells) usually increases with the dose of the chemical.

Preliminary experiments with the V79 cells showed that diesel extracts at 100 μg/mL (μg DPE/ml culture medium) were fairly toxic, but enough viable cells could be recovered after the treatment to determine the mutation frequency. This dose was therefore chosen as the most likely dose for detecting mutagenic activity. A lower dose, 50 μg/mL, was usually much less toxic and was also tested in some experiments. The toxicity was quantitated by the cloning efficiency of the cells after treatment. The values are shown for the experiments in Tables 2, 3, and 4.

The cloning efficiency of V79 cells was also measured after exposure to additional concentrations of the extract DPE(2). The results of this experiment are shown in Fig. 1. There is a dose-dependent decrease in the viability of the cells at concentrations between 25 and 100 μg DPE/mL culture medium; at 25 μg/mL or less there is little or no difference from the control cells. The cytotoxic effect is visible microscopically as cells exposed to 100 μg/mL of extracts change from the normal flat, irregular shape to a round ball which is more loosely attached to the culture dish (Fig. 2).

Mutagenicity

The initial mutagenicity experiments with the V79 cells investigated the activity of DPE(1), the extract of diesel particles collected by filtration. The results with this sample are shown in Tables 2 and 3. The method reported by Chu [5] was followed in the first experiments, using the selection agent 8-azaguanine. In Table 2, experiment A, MNNG has induced the frequency of the azaguanine-resistant cells over the spontaneous frequency seen in cells exposed only to DMSO. B[a]P, which requires activation, and DPE(1) have no effect. In experiment B, the mutation frequency of cells exposed to B[a]P and S9 appears to be increased, but DPE(1) at 50 μg/mL culture medium has no significant effect with or without S9.

In Table 3, results of one experiment comparing three different selection agents are shown. The response to three concentrations of MNNG was investigated. Duplicate flasks were treated with each condition to compare the variability within a particular experiment. The exposure time was also lengthened to 16 hours, to increase the possibility of detecting a less active compound.

No distinct colonies were detected in the control plates (DMSO-treated). The frequency is calculated as less than 1 mutant per total number of surviving cells (at least 5 x 10^5 cells for each treatment). The mutations induced by MNNG show a dose-dependent response for all selection agents, even though the initial toxicity was high. The values of the duplicate plates are close in most cases. DPE(1) at 50 μg/mL culture medium showed no activity at all; at 100 μg/mL, no colonies were selected with 6-TG while a few were apparent with 8-AG and ouabain. The resulting frequency

TABLE 2 PRELIMINARY STUDIES:

INDUCTION OF 8-AZAGUANINE RESISTANCE IN V79 CELLS

Expt.	Conditions*	Dose (μg/ml culture medium)	S9 (0.8%)	Cloning Efficiency (%)	8-AG-resistant colonies/10^5 cells†
A	DMSO-1%	-	-	77	2.2
	DPE(1)	100	-	13	0.8
	B[a]P	2	-	74	0.2
	MNNG	2	-	34	30.4
B	DMSO-0.5%	-	-	67	1.0
			+	71	0.2
	DPE(1)	50	-	52	0.2
			+	57	1.4
	B[a]P	2	-	57	0.2
			+	52	5.2

*Treat 4 x 10^6 cells/25 cm^2 flask/condition in DME for 5 hour exposure period.

†Selection agent 8-azaguanine (30 μg/ml) added 10 days (A) or 11 days (B) after exposure period. Cells subcultured days 1, 4, and 7 (A) or days 2, 4, 6, and 9 (B) after exposure period. A total of 5 x 10^5 cells/condition were plated at the final subculture for selection of resistant colonies.

TABLE 3 COMPARISON OF SELECTIVE AGENTS: OUABAIN, 8-AZAGUANINE, AND 6-THIOGUANINE

		#Resistant Colonies/10^5 Surviving Cells†		
Conditions*	Cloning Efficiency (%)	Ouabain	8-Azaguanine	6-Thioguanine
DMSO - 1%	86 -	< 0.2 < 0.2	< 0.2 < 0.2	< 0.2 < 0.2
MNNG 0.5 μg/ml	32	7.3	8.0	4.2
MNNG 1.0 μg/ml	10.2 13.4	18.4 15.3	26.2 23.8	19.8 12.4
MNNG 2.0 μg/ml	3.6 3.2	44.4 51.4	40.6 43.6	22.4 33.0
DPE(1) 50 μg/ml	63 49	< 0.2 < 0.2	< 0.3 < 0.3	< 0.3 < 0.3
DPE(1) 100 μg/ml	13 16	3.5 5.3	0.3 1.2	< 0.3 < 0.3

*Plated 10^6 cells/25 cm^2 flask, 2 flasks/condition. Cells were exposed for 16 hours to MNNG (0.5-2 μg/ml culture medium) or DPE (50-100 μg DPE/mL culture medium).

†At the end of the exposure period, the V79 cells were plated for selection with ouabain after a 2 day expression period or with 8-azaguanine and 6-thioguanine after a 6 day expression period.

TABLE 4   EFFECT OF DPE(2) ON MUTATIONS TO OUABAIN RESISTANCE

Conditions*	Cloning Efficiency (%)	Ouabain Resistant Colonies	Mutation Frequency/10^5 Surviving Cells
Control (1% DMSO)†	92	1	0.03
DPE(2) - 75 μg/ml†	34	1	0.1
DPE(2) - 100 μg/ml††	35	1	0.05
MNNG - 2 μg/ml††	3	323	153

*Plated 3 x 10^6 cells/75 cm^2 flask/condition 7 hours before exposure period. Treated for 16 hours, then subcultured for expression and cloning efficiency. Ouabain was added after 2 days for selection of resistant colonies.

†Plated 3 x 10^6 cells/30 culture dishes for selection; 500 cells/5 dishes for cloning efficiency.

††Plated 6 x 10^6 cells/30 culture dishes for selection; 1000 cells/5 dishes for cloning efficiency.

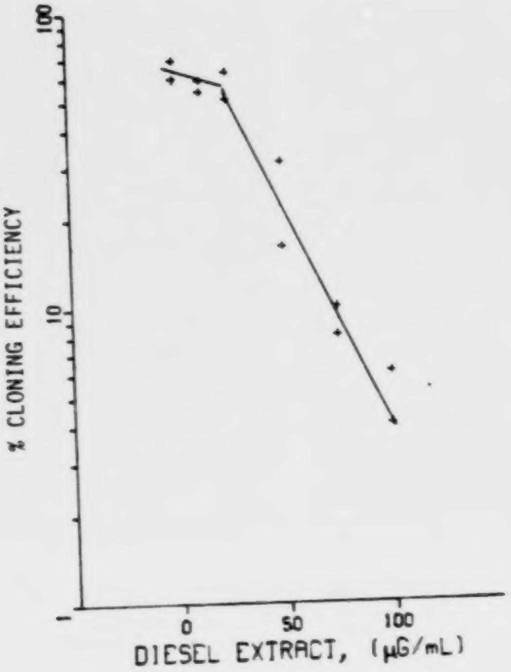

Figure 1 Cytotoxicity of diesel extract for Chinese Hamster cells. Cells were treated for 16 hours with various concentrations of DPE(2) expressed in μg DPE/mL culture medium, as described in Methods. Each marker indicates the cloning efficiency of 400 treated cells.

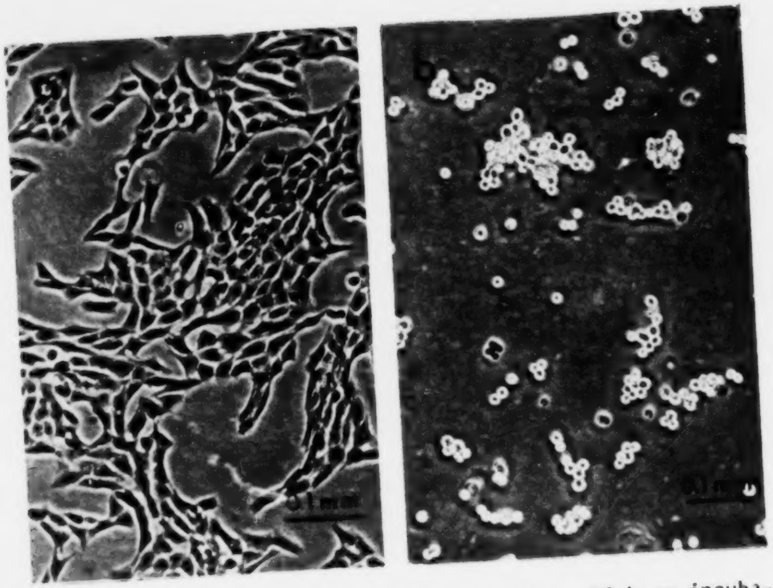

Figure 2 Appearance of V79 cells after 16 hour incubation with or without diesel extract. Cells were plated (2.7×10^6 cells/75 cm^2 flask) 6 hours before addition to medium containing (a) 1% DMSO or (b) 100 μg DPE(2)/mL culture medium.

with 8-AG is still lower than what might occur spontaneously (see Table 2), while the result with ouabain is higher than expected.

For the remaining experiments, a dichloromethane extract of diesel particles collected by electrostatic precipitation, DPE(2), was obtained. Its effect on mammalian cells was first investigated using ouabain as the selective agent (Table 4). No induction of mutations by DPE(2) was seen, with only one colony recovered from more than 10^6 surviving cells. MNNG, however, was very active.

Fig. 3 summarizes several separate experiments comparing the frequency of thioguanine-resistant colonies in V79 cells exposed to the test solvent DMSO (1%), DPE(2), benzo[a]-pyrene, or MNNG. S9, a supernatant of 3-methylcholanthrene-induced rat liver homogenate, was added to investigate the possibility that some substances in the diesel may be activated by enzymes not present in the cells. B[a]P was tested as a positive control for activation. The results show that cells treated with the diesel extract are not different from the solvent-treated cells either with or without addition of S9. Although not shown, concentrations of 50 μg/mL diesel extract also produced no increase in mutations. B[a]P increases the mutation frequency when in the presence of S9, although it is much less effective than MNNG.

Microsomal Enzymes and Diesel Extract Cytotoxicity

Although the diesel extract with added S9 was not mutagenic to the V79 cells, the S9 did appear to reduce the toxicity of high concentrations of the extract, even though the S9 itself was toxic. To verify this observation, the cloning efficiency of the V79 cells was measured after treatment with DPE(2), 0 to 100 μg/mL, in the presence or absence of S9. The possibility of adsorption versus enzymatic conversion was investigated by comparing S9-treated cells with and without the cofactors NADP and glucose-6-phosphate, required by most metabolizing enzymes. The results of this experiment (Fig. 4) confirmed the initial observations that with S9 plus cofactors, there is little increase in toxicity from low to high concentrations of diesel extract and that S9 addition is responsible for the largest amount of cell death. Adsorption of toxic components in the extract to the lipid and protein components in the S9 may provide some of the protection, as cell survival was increased with S9 alone. Addition of the cofactors further reduced the toxicity of the extract, implying that the metabolizing enzymes are also important.

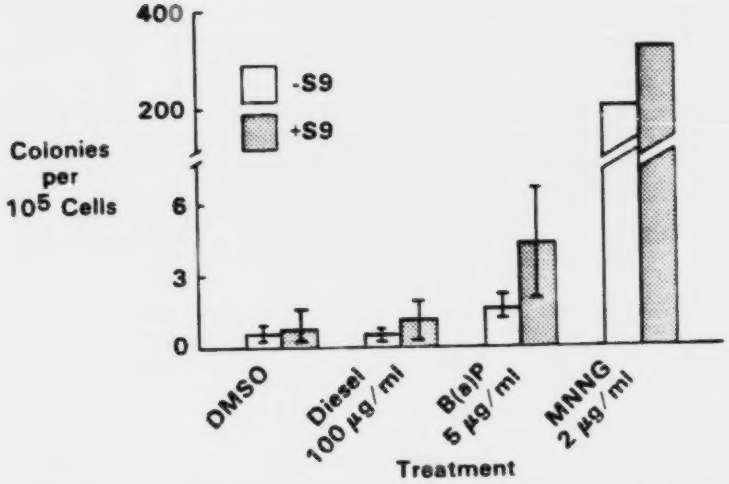

Figure 3 Effect of diesel extract DPE(2) on the frequency of 6-thioguanine resistant colonies. The mutation frequency was compared for cell populations treated with DMSO (1%), diesel extract, benzo[a]pyrene, or MNNG, with or without the addition of S9 (20 µL/mL) as indicated. Cells were initially plated (3 x 10^6 cells/75 cm^2 flask) 6 hours before treatment period of 16 hours. The expression period was 8 days. Cells were treated with 6-TG after being plated two days previously at 5 x 10^4 cells/100 mm dish, using total of $\sim10^6$ cells/condition/experiment.

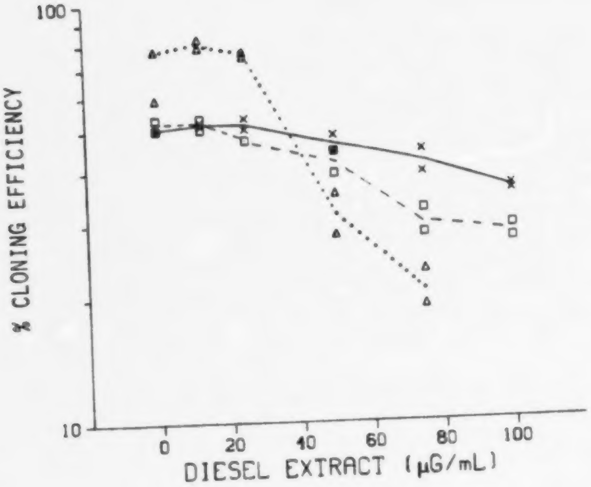

Figure 4 Comparison of the cytotoxicity of diesel extract DPE(2) with and without S9 and enzymatic cofactors. Cells were incubated for 16 hours in their normal culture medium (Δ) or in medium containing 20 μl/ml S9 with (x) or without (□) the cofactors NADP and glucose-6-phosphate. After the treatment period, the cells were analyzed for their cloning efficiency as described in Figure 2.

Mouse Lymphoma Cell Assay

The results with the V79 cells were supported by studies performed on a similar sample by Litton Bionetics Laboratory in the mouse lymphoma cells (Fig. 5). The growth of the cells in normal medium after treatment with the diesel extract was initially inhibited when compared with the untreated cells. This is an indication of the toxic effect of the treatment, a combination of cell death and reduction in the growth rate. The presence of S9 in the culture medium reduces the toxicity of the extract to the extent that about twice as much extract is required to produce the same effect. This enables higher concentrations to be tested in the presence of S9 as a sufficient number of surviving cells can be obtained for mutagenicity testing.

As reported by the testing laboratory, the results of the mutagenicity tests show no increase in the frequency of mutant colonies in the non-activated system (-S9) at doses up to 140 μg/mL, but a relatively small increase at about 200 μg/mL. Similar values are obtained with identical concentrations in the presence of S9 indicating little or no activation of the extract. Higher concentrations of the extract, which were tested only in the presence of S9, caused an increase in the mutation frequency. However, this may not be the result of normal enzymatic activation of the polycyclic aromatic hydrocarbons in the extract. In the presence of 400 μg/mL of diesel extract, the benzo[a]pyrene hydroxylase activity of rat liver microsomes is reduced to 95% of the initial level [7].

DISCUSSION

The diesel extracts have so far proven negative as mutagens of V79 cells. However, because the extracts are a complex mixture of substances, the toxicity may be inhibiting the demonstration of mutagens. In the mouse lymphoma mutagenicity assay, activity of similar diesel extracts is negative at concentrations up to 140 μg/mL. Concentrations greater than 200 μg/mL could be tested only in the presence of S9 which reduced the toxicity without blocking the mutagenic activity. Both studies indicate that the mutagenic effects of the diesel extracts are relatively weak in mammalian cells. Therefore, the effects may not occur *in vivo* until the dose of deposited diesel particulates in the organism is manifested by the concurring cellular toxic effects.

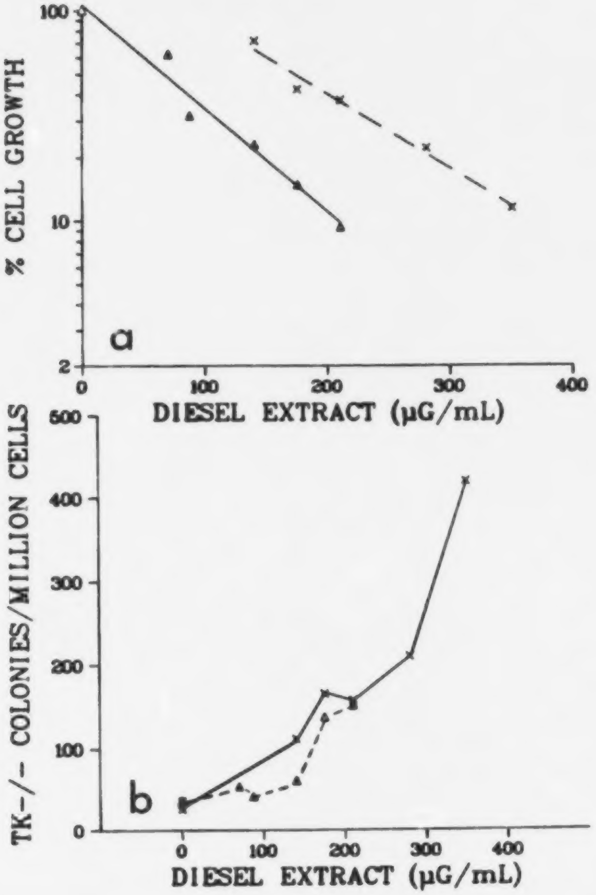

Figure 5 Cytotoxicity and mutagenicity of diesel extract in mouse lymphoma L5178Y cells. Various concentrations of a dichloromethane extract of diesel particles in culture medium were incubated for 4 hours with cells in suspension culture in the presence (x) or absence (Δ) of S9. After removal of the test medium, toxicity was evaluated by the relative amount of cell growth in the next 24 hours (a). Mutagenicity was evaluated by determination of the frequency of thymidine kinaseless (TK^-/-) cells after a two day expression period (b).

ACKNOWLEDGEMENTS

I wish to thank J. Dickman for providing excellent technical assistance and T. L. Chan, J. D'Arcy, and J. T. Johnson for collection of the diesel samples and preparation of the extracts.

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General Discussion

F. DANIEL: When you were trying to titrate out the cytotoxicity by using S9 did you ever try just boiling the S9 instead of leaving out cofactors?

C. RUDD: I thought about using heat inactivated S9 as a control for absence of activation, but it precipitated badly. I did do it, but it was a mess because of the precipitate and I didn't consider it a real control.

DIESEL SOOT: MUTATION MEASUREMENTS IN BACTERIAL AND HUMAN CELLS

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ABSTRACT

Both hot pipe and dilution chamber samples of the exhaust from a diesel (Oldsmobile 350) engine have been collected, extracted with methylene chloride and those extracts have been tested for mutagenicity in forward mutation assays in human lymphoblasts and S. typhimurium. In the absence of a metabolic activation system, the extract was significantly mutagenic to the bacteria in the range of 0 to 30 μg/ml, but induced no mutations in human cells at concentrations up to 200 μg/ml under the same conditions of assay medium. However, when assayed in the presence of a postmitochondrial supernatant derived from rat liver, the soot extracts were significantly mutagenic to both bacteria and human cells in the range of 50-100 μg/ml. Fractionation of the soot extract on the basis of polarity by sequential elution from a silicic acid column permitted concentration of the mutagenic activity in the alkane/toluene eluate, as determined by bacterial assays. Preliminary characterization of this fraction and preliminary studies of pure compounds leads

us to suspect the alkyl substituted anthracenes and phenanthrenes as representing at least a significant fraction of the mutagenic activity of this alkane/toluene eluate.

INTRODUCTION

Based on our studies of kerosene soot mutagenicity in bacteria (Kaden et al., 1979) and human cells (Skopek et al., 1979), we have approached the study of diesel engine emissions with a hypothesis that its mutagenicity (if any) may be accounted by the summation of the products of concentrations and specific mutagenic activities of the soot's individual chemical components. Thus, our approach requires a close collaboration between an analytical chemistry laboratory at Indiana University and a genetic toxicology laboratory at M.I.T.

We have only begun this chemo- and bioanalytical approach to diesel soot in the last six months, and wish to report our findings to date for unfractionated methylene chloride extracts and some broad fractionations based on polarity.

METHODS

Sample sources:

Dr. Charles Hobbs of the Inhalation Toxicology Research Institute, Albuquerque, New Mexico supplied our first diesel exhaust sample which was taken on a glass fiber filter by a hot pipe sampling approach. No attempt to calibrate engine load or performance was made in this preliminary sampling of an Oldsmobile 350 engine burning a commercial diesel fuel. Dr. Morton Beltzer of Exxon Research and Engineering Company, Linden, New Jersey, substantially helped our project by supplying us with a 2 gm single sample of diesel soot extract. An Oldsmobile 350 engine burning blended typical refinery diesel fuel products similar to commercial No. 2 fuels was operated with repetitive hot start Federal Test Procedures. The exhaust was passed through a dilution chamber and collected on a Pallflex type T60A20 filter, 36 inches square. The filter was extracted overnight with methylene chloride in a soxhlet extractor. The solvent was evaporated on a steam bath with nitrogen purge. The sample was shipped on dry ice and has been stored by us at -80° C.

Mutation assays:

Bacterial mutation to 8-azaguanine resistance was performed as described in Kaden et al., 1979. Human cell mutation to both 6-thioguanine resistance and trifluorothymidine resistance was measured as described in detail in Thilly et al., 1980.

Source of post mitochondrial supernatant:

Liver post mitochondrial supernatant from aroclor pretreated rats was purchased from Litton Bionetics, Rockville, Maryland. Materials was received frozen on dry ice and stored by us at -80°C. This preparation was consistently contaminated with several bacteria or fungi per 5 milliliter aliquot necessitating sterilization by a 1 megarad gamma ray exposure (in dry ice). This exposure does not affect the ability of the PMS to metabolize benzo(a)pyrene to a mutagen in bacteria or human cells.

RESULTS

Figure 1 reports our measurement of the mutagenicity of the two diesel extracts to bacteria, and of the dilution chamber sample to human lymphoblasts when they were coincubated with the extracts for two hours in the absence of any added metabolizing system. Note that both samples were potent mutagens for the bacteria, but no mutagenic activity toward human cells was detected. In a subsequent experiment, bacteria were coincubated with the extracts in the presence of standard cell culture medium; the amount of bacterial mutation was only slightly reduced by this change in exposure medium.

Figure 2 reports our observations when the experiments were repeated in the presence of a rat liver post mitochondrial supernatant metabolizing system with cofactors and pH conditions appropriate for observing maximum mutagenicity from 40 μM benzo(a)pyrene. Exposures were for two hours; the post mitochondrial fraction was 10% v/v in the bacterial studies and 5% v/v in the human cell studies. Under these conditions, the amount of mutation induced in human cells and bacteria is quite similar with the 99% upper confidence limit on historical controls being exceeded at about 50 μg/ml for the human cells and at about 100 μg/ml for the bacterial cells. This similarity of sensitivity has been observed by us for other combustion residues such as kerosene soot and pure polycyclic aromatic hydrocarbons (Kaden et al., 1979 and Shopek et al., 1979).

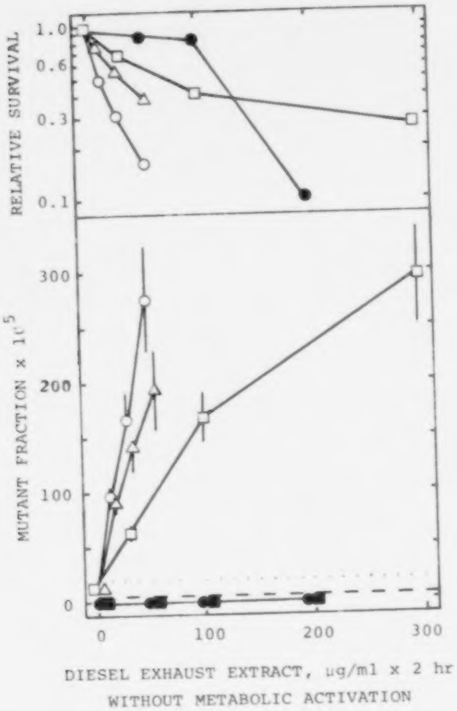

Figure 1. Toxicity and Mutagenicity of Diesel Exhaust Extracts to Salmonella typhimurium and Human Lymphoblasts in the Absence of Metabolic Activation.

Bacteria or human cells were treated for 2 hr with diesel exhaust extracts. Relative survival was determined by cloning efficiency immediately after treatment. Mutant fractions were determined for 8-azaguanine resistance (50 µg/ml) in bacteria, and 6-thioguanine resistance (5 µg/ml) or trifluorothymidine resistance (1 µg/ml) in human cells. Error bars are 99% confidence intervals. The dotted line is the 99% upper confidence limit for the historical background mutant fraction in bacteria; the dashed line is the 99% upper confidence limit for the background mutant fraction in human cells.

○ - Bacteria treated in minimal media - Hot pipe sample
△ - Bacteria treated in complex media - Hot pipe sample
□ - Bacteria treated in minimal media - Dilution chamber sample
● - Human cells, trifluorothymidine resistance - Dilution chamber sample
■ - Human cells, 6-thioguanine resistance - Dilution chamber sample

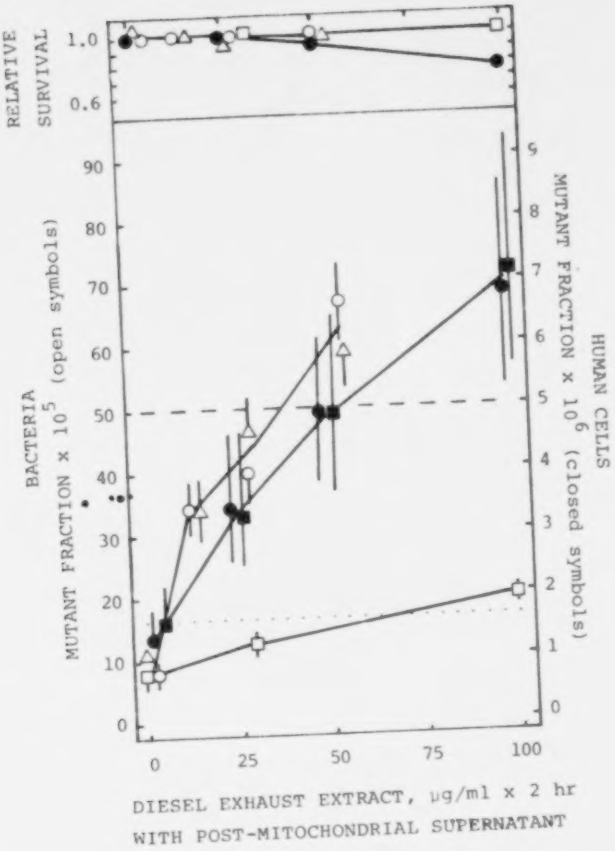

Figure 2. Toxicity and Mutagenicity of Diesel Exhaust Extracts to Salmonella typhimurium and Human Lymphoblasts in the Presence of Metabolic Activating System.

Bacteria or human cells were treated for 2 hr with diesel exhaust extracts in the presence of 10% (bacteria) or 5% (human cells) aroclor-induced rat liver post-mitochondrial supernatant.

○ - Bacteria treated in minimal media - Hot pipe sample
△ - Bacteria treated in complex media - Hot pipe sample
□ - Bacteria treated in minimal media - Dilution chamber sample
● - Human cells, trifluorothymidine resistance - Dilution chamber sample
■ - Human cells, 6-thioguanine resistance - Dilution chamber sample

Fractionation of the crude extracts was followed by bacterial mutation assay of each fraction. No human cell assays have been performed on the fractions at this time. As summarized in Table 1A, the most active fraction of the ITRI hot pipe sample, both with and without metabolic activation, was the one eluted from the silicic acid column by a 1:1 pentane:toluene solvent after the column had been rinsed with pentane. Similarly, for the Exxon dilution chamber sample, the hexane:toluene fraction was the most mutagenic fraction in the presence of a metabolic activating system. In the absence of metabolism, the toluene fraction appeared to be more mutagenic; however, because of the extreme toxicity of this fraction, the data for this fraction is considered preliminary. One may note that the amount of mutaenic activity recovered from this column separation was approximately equal to the amount originally loaded on the column.

DISCUSSION

Insofar as gene locus mutation assays may be predictive of human health risk, our observations lead us to consider diesel engine exhausts with grave concern. It is our present intention to continue our chemical analysis of the sample obtained from the dilution chamber and to test the compounds identified in it until we have accounted for the biological activity in terms of individual mutagens. We hope that our studies will help combustion scientists to focus on those compounds and compound classes which must be reduced in diesel exhausts if their mutagenic potencies are to be reduced.

We note that our studies with human cells indicate a possibly lesser priority for immediate studies of so-called 'direct acting' mutagens since these were observed in bacterial but not in human cell mutation assays. We intend to follow this preliminary conclusion in setting our research priorities for the coming year.

Finally, we must state our concern that without knowledge of the physiologic distribution of inhaled soot particles, there seems to be no logical means to use the kind of observations made in our studies to estimate the impact on human health. Until such knowledge is obtained by competent scientists, predictions based on analytical logic do not seem possible.

Table 1A

MUTAGENICITY OF FRACTIONS OF THE HOT PIPE SAMPLE OF DIESEL EXHAUST TO S.TYPHIMURIUM

Fraction	% Weight of Unfractionated Mixture	Concentration tested (μg/ml)	$8\ AG^R$ MF x 10^5 ± 99% CI* No Activation	$8\ AG^R$ MF x 10^5 ± 99% CI* 10% ARO PMS
Soot (minE)	100	13	95 ± 12	34 ± 4
		27	165 ± 25	40 ± 4
		53	280 ± 54	67 ± 6
Soot (RPMI)	100	13	90 ± 12	34 ± 5
		27	141 ± 22	47 ± 5
		53	190 ± 31	59 ± 6
Pentane	18	4.8	5 ± 2	7 ± 2
		9.5	4 ± 1	8 ± 2
		19	5 ± 1	11 ± 2
Pentane: Toluene	13	3.3	42 ± 5	32 ± 5
		6.5	107 ± 13	48 ± 8
		13	235 ± 43	98 ± 14
Toluene	7	1.8	28 ± 4	---
		3.5	40 ± 5	13 ± 2
		7.0	78 ± 9	31 ± 4
Methylene Chloride	8	2	23 ± 3	8 ± 2
		4	30 ± 4	---
		8	56 ± 7	21 ± 3
Methanol	54	14	16 ± 2	7 ± 2
		28	23 ± 3	6 ± 2
		56	35 ± 4	9 ± 2
Control	---	0	4 ± 1	7 ± 2

Legend: * 8-azaguanine-resistant mutant fraction, with the 99% confidence interval.
Assays were performed without activation or in the presence of 10% (volume/volume)
aroclor induced rat liver post-mitochondrial supernatant (10% ARO PMS).

Table 1B

MUTAGENICITY OF FRACTIONS OF THE DILUTION CHAMBER SAMPLE OF DIESEL EXHAUST TO S.TYPHIMURIUM

Fraction	% Weight of Unfractionated Mixture	Concentration tested (μg/ml)	8 AG^R MF x 10^5 ± 99% CI*	
			No Activation	10% ARO PMS
Soot (Unfractionated)	100	30	67 ± 7	12 ± 2
		100	167 ± 22	19 ± 2
		300	295 ± 45	39 ± 3
Hexane	27	150	55 ± 7	67 ± 7
		300	71 ± 8	91 ± 9
Hexane: Toluene	7	150	516 ± 143	203 ± 23
		300	---	---
Toluene	7	150	~ 1000	105 ± 11
		300	---	172 ± 20
Methylene Chloride	14	150	337 ± 60	50 ± 5
		300	134 ± 100	73 ± 8
Methylene Chloride: Methanol (2:1)	38	150	92 ± 12	---
		300	149 ± 21	35 ± 4
Methylene Chloride: Methanol (1:2)	4	75	9 ± 2	7 ± 1
		150	14 ± 2	8 ± 1
Methanol	4	75	5 ± 1	9 ± 2
		150	7 ± 1	7 ± 1
Control	-	0	10 ± 2	5 ± 1

Legend: * 8-azaguanine-resistant mutant fraction, with the 99% confidence interval. Assays were performed without activation or in the presence of 10% (volume/volume) aroclor induced rat liver post-mitochondrial supernatant (10% ARO PMS).

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Discussion

A. BROOKS: Do I understand that all of the direct-acting mutagenic activity we see in bacteria is of no consequence in humans?

W. THILLY: Up to a constant of 200 micrograms per milliter in the culture; the answer to that question is yes.

STUDIES ON THE EFFECTS OF DIESEL PARTICULATE ON NORMAL AND XERODERMA PIGMENTOSUM CELLS

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ABSTRACT

Diesel engine emission particulates (DP) have been shown to contain direct acting bacterial mutagens. Our investigations have shown that normal human fibroblasts (NF) and excision repair deficient *xeroderma pigmentosum* figroblasts (XP) (neither of which possesses significant amounts of the cytochrome P448-P450 metabolizing system) are sensitive to the cytotoxic effects of the direct-acting agent(s) of DP. XP fibroblasts show greater sensitivity than NF to DP suspensions in DMSO or organic solvent extracts of DP. When these cell strains are exposed to equal amounts of DP extracts, the slope of the XP survival curve is at least twice as steep as that of NF. More significantly, there is a pronounced shoulder on the survival curve. We also observe this difference in survival when these strains are treated with polycyclic aromatic hydrocarbons and several other chemicals and have demonstrated that it is indicative of tghe formation of lethal DNA aducts for which XP cells have little or no repair capacity. We have found that short extractions of DP with cold acetone, cold CH_2CL_2, warm toluene, or a 4-hour Soxhlet CH_2CL_2 extraction are equally effective in solubilizing the direct-acting cytotoxic component(s) of DP. Biological fluid extracts of DP employing Ham's F10 Nutrient Medium or fetal calf serum also contain cytotoxic chemicals. Howver, these extracts were

only 1/6th to 1/27 as cytotoxic as DMSO suspensions of the DP. HPLC fractionation on a Bio-Rad silica (20 44 u) column of Soxhlet-CH_2CL_2 extracts of DP has provided a single cytotoxic fraction. However, since humans are exposed to whole diesel particles rather than organic solvent extracts of them, it seemed desirable to examine the effect of diesel particulate itself on human fibroblasts. We found that when NF and XP cells were exposed directly to suspensions of DP in tissue culture medium containing serum, the cytotoxic effect was virtually identical to that found to organic extracts of the same DP sample. This result suggests that the differentially cytotoxic (DNA damaging) material on the particles was somehow able to transfer from the DP and come into contact with the DNA. Electron microscopy of cells exposed directly to DP demonstrated that the cells contained large amounts of DP. Our previous studies have shown that agents which cause higher cytotoxicity in XP than in NF cells also induce mutations. Therefore, diesel exhaust particulate represents a potential health hazard for man. Whether it represents an actual hazard can only be determined by further study.

General Discussion

R. CHRISTIAN: I was very interested in your studies of bioavailability and putting particles on cells. We have not done this with diesel exhaust but we have with coal particles ground very finely. When you put the particles on cells, they do coat the cells and are taken in. This can be seen with electron microscopy and you see phacocytosis in normal tissue cultured cells. The serum as well as the tissue culture medium is quite effective at leaching toxic substances. One of the real advantages here is that you can treat your medium with the particles that you wish to use and grow your cells in it. I think that this is quite an advantage.

J. MCCORMICK: Well, we think that this work is actually modelled on your studies, and the results I showed indicate that the tissue culture medium, or serum, are very poor leachers of the material that is differentially cytotoxic in these cells. So that doesn't seem to be a viable way to go here.

R. CHRISTIAN: You do observe the particles associated with the cells and in cases they are taken inside. Were you suggesting that perhaps there was some phacocytosis there?

J. MCCORMICK: Yes, that is the most likely explanation. We haven't done the electron microscopy yet to demonstrate it, but I suspect that that is exactly what happened.

R. CHRISTIAN: I would like to ask you whether you have considered the possibility of these extracts being promoters as well as initiators? It seems that with the prospect of the exhaust being in our environment that promotion may also be a problem seen in the future.

J. MCCORMICK: Promotion is certainly worth looking at, but we have not looked at it in our studies.

F. DANIEL: I noticed on your HPLC profiles that you had identified several fractions having activity. Have you gotten anywhere on identifying the chemical components on those fractions?

J. MCCORMICK: No, we haven't really done that yet.

A. BROOKS: As I understand it, you really don't have any direct evidence of interaction with the DNA in these cells. Because of that, I wonder if you have tried to get the differential toxicity using some of the fractions that are very cytotoxic. If after making fractionation, you find that some of the toxicity is associated with fractions that are nonmutagenic in the Ames Test, I wonder if you tested any of those non-mutagenic, but cytotoxic fractions?

J. MCCORMICK: No, we have not done that.

BENZO(A)PYRENE ALTERS LUNG COLLAGEN SYNTHESIS IN ORGAN CULTURE

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ABSTRACT

Benzo(a)pyrene is known to be a significant component of diesel emissions. In our studies, benzo(a)pyrene markedly altered parameters of collagen synthesis in organ cultures of rat lungs.

Cultures exposed to benzo(a)pyrene synthesized greater amounts of type III in relation to type I collagen. Exposure to benzo(a)pyrene also markedly elevated prolyl hydroxylase activity in lung organ cultures. No significant changes occured in cultures treated with pyrene, a non-carcinogenic hydrocarbon.

Collagen plays an important role in lung function, and in regulating cell differentiation and proliferation. Alteration of collagen synthesis appears to be an important aspect of the toxicity of benzo(a)pyrene.

INTRODUCTION

Collagen is an integral part of lung structure, contributing up to 20% of the dry weight, and it plays a major role in pulmonary function (1). Qualitative and quantitative changes in collagen, aberrations in collagen synthesis, and altered patterns of tissue distribution of collagen are major aspects of induced lung disease (2-4). Malignant changes in lungs are often accompanied by morphologically observed changes in collagen (5,6). Carcinogenesis alters the structural and functional characteristics of tissues, and changes in collagen synthesis may be expected to be a part of the early events in carcinogenesis.

Collagen is a regulatory protein, controlling vital aspects of cell function and differentiation by mediating cell-cell interaction and cell adhesion, and it regulates cell proliferation (7). Since benzo(a)pyrene, a carcinogenic polyaromatic hydrocarbon, is a component of diesel emissions, it is of interest to determine if it affects lung collagen synthesis.

In another report from our laboratory presented at this symposium (8), we reported the alteration of collagen synthesis in lungs of rodents exposed to diesel emissions. In the present study, we investigated the effect of benzo(a) pyrene and pyrene on collagen synthesis in organ cultures of rat lung. While benzo(a)pyrene markedly altered parameters of collagen synthesis, no such changes were observed in cultures exposed to the non-carcinogenic hydrocarbon pyrene.

MATERIALS AND METHODS

Radiochemicals

L-[3,4-^{3}H]-proline, 25 Ci/mmol, and L-[^{14}C(U)]-proline, 285 mCi/mmol were purchased from New England Nuclear.

Organ Culture Procedures

Lung organ cultures were prepared by a method developed in our laboratory, and described in detail elsewhere (9). The method consists of placing 1 mm thick slices of neo-natal rat lung (Long-Evans) on Millipore filters (0.3 m pore size, 2.5 cm diameter), supported on 1.0 ml of Dulbecco-Vogt minimum essential medium containing 10% fetal calf serum, in an organ culture dish (Falcon Plastics). Several dishes hydroxylase (16). The increase in aryl hydrocarbon hydroxylase in this system was consistent with previous in vivo and in vitro studies on the toxicity of this compound. An additional aspect of chemical injury elicited by benzo(a) pyrene in lung organ cultures was expressed as a large increase in prolyl hydroxylase activity, paralleling the increase in aryl hydrocarbon hydroxylase (16). Increased tissue prolyl hydroxylase activity reflects tissue injury and connective tissue proliferation (5,6,25).

When cultures were maintained in the presence of 10 μm or 25 μm benzo(a)pyrene for 12 hours, the activity of prolyl hydroxylase was increased by nearly 80% (Table I). These data indicated that benzo(a)pyrene may induce increased collagen synthesis in lung organ cultures. There was no increase in pyrolyl hydroxylase activity in cultures exposed to 25 μm pyrene, a structurally related hydrocarbon with low carcinogenic potential.

The synthesis of collagen was examined in these cultures by pulse-labelling with ^{14}C-proline. The cultures were maintained in the presence or absence of benzo(a)pyrene for 24 hours, and ^{14}C-proline added for a 3-hour pulse period. As seen in Table II, benzo(a)pyrene caused a significant increase in the specific activity of ^{14}C-hydroxyproline synthesized, confirming an increased collagen synthesis. There was no significant difference between control cultures and cultures exposed to pyrene.

Collagen is a famil of genetically distinct proteins (26). The proportion of different types of collagen vary from tissue to tissue, and change during growth and development, and in pathological conditions (27). Type I collagen is ubiquitous and is the major collagen component of adult lungs. Type III collagen chains are predominant in fetal tissues, and are associated with rapidly proliferating tissues, and the proportion of type III collagen synthesis is markedly increased in regenerating tissues and scars.

As seen in Table III, the synthesis of type I collagen accounted for 70% of the total collagen syntehsized in the control cultures. Type III collagen accounted for the remaining incorporation. In marked contrast, type III collagen accounted for nearly half of the total collagen synthesized in cultures exposed to benzo(a)pyrene. These data suggest that benzo(a)pyrene induces chemical injury resulting in increased expression of a fetal gene product, namely type III collagen.

The present studies do not provide information concerning the mechanism of chemical injury induced by benzo(a)pyrene were placed in a humidified, gas-tight chamber in which a gas mixture, 95% air + 5% CO_2, was circulated. Benzo(a) pyrene and pyrene were dissolved in acetone before, for addition to the cultures. Control cultures received the same volume of acetone (2 1). Previous experiments demonstrated that the addition of this quantity of acetone had no biochemically or morphologically observable effects on the organ cultures.

Analytical Procedures

The DNA content of the cultures was determined by the diphenylamine procedure (10). Total protein content was assayed by measuring the ninhydrin reactive material in the hydrolyzates of the tissue, and expressed in terms of "leucine equivalents" (11). Collagen synthesis was determined by following the incorporation of radioactivity from proline into radioactive hydroxyproline, assayed by a published method (12). Prolyl hydroxylase activity was determined in the 15,000 x g supernatants of the cultures by previously published methods (13), and expressed in terms of 3H-proline-labeled, unhydroxylated collagen substrate per mg of tissue DNA.

Collagen Chair Analysis

The chain composition of newly synthesized collagen was determined as described elsewhere, in order to determine the relative incorporation of radioactivity into type I and type III collagens (14).

RESULTS AND DISCUSSION

Lung organ cultures have been shown to be useful for studies on lung collagen metabolism under a variety of conditions, including hyperoxic atmospheres, and in the presence of several agents known to be injurious to lungs *in vivo* (14-20). Lung organ cultures have also been useful in investigations on the effects of chemical carcinogens (21-24).

Exposure of lung organ cultures to hyperoxic environments (14), paraquat, or heavy metals (16-20) elicited marked increases in the rates of collagen synthesis. The biochemical and morphological response to chemical injury in lung organ cultures mimicked the response of lungs *in vivo*. Exposure of lung organ cultures to benzo(a)pyrene also caused a marked increase in the levels of aryl hydrocarbon

Table I

EFFECT OF BENZO(A)PYRENE AND PYRENE ON PROLYL HYDROXYLASE ACTIVITY IN LUNG ORGAN CULTURES

Culture Conditions	Prolyl Hydroxylase Activity 10^{-4}xdpm ^{3}HHO Released/mg DNA	% Change
Control	6.44	--
Benzo(a)pyrene, 10 µm	10.81	+ 67
Benzo(a)pyrene, 25 µm	11.45	+ 77
Pyrene, 25 µm	5.57	+ 3

Each number is the average of five determinations. Enzyme specific activity was calculated on the basis of the DNA content in order to relate it to the cellularity of the tissue.

Table II

EFFECT OF BENZO(A)PYRENE ON COLLAGEN SYNTHESIS IN LUNG ORGAN CULTURES

Culture Conditions	10^{-5}xdpm ^{14}C-HyP/mMole LE	% Change
Control	2.80	--
Benzo(a)pyrene, 10 µm	3.41	+ 22
Benzo(a)pyrene, 25 µm	3.76	+ 34

Culture conditions are described in the Methods Section. Tissues were maintained under the described conditions for 21 hours, and then pulse-labelled with ^{14}C-proline for 3 hours.

LE = Leucine Equivalents.

which contributes to increase connective tissue proliferation. However, it is known that the metabolism of carcinogenic hydrocarbons such as benzo(a)pyrene, results in the generation of many free radical species, including the oxygen-free radical anion superoxide (28). In contrast, the metabolism of noncarcinogenic hydrocarbons, including pyrene, does not result in a comparable flux of free radicals. Superoxide free radicals has been shown to be a major component of tissue toxicity of oxidants and other injurious agents (29). Other studies in our laboratory have shown that superoxide induces enhanced collagen synthesis in lung fibroblast cultures (30), in cultures of hepatocytes (31), and is the collagen-enhancing agent in the toxicity of paraquat in lungs (15).

In our studies reported here, the syntehsis of collagen was altered after short exposures to benzo(a)pyrene. In this short period, cell transformation cannot be considered to be a major factor for the altered biochemical benhavior of the tissue, although this possibility cannot be entirely ruled out. Type III collagen is always associated with rapidly proliferating cells and tissues, and there is some evidence suggesting that type III collagen regulates cell proliferation and differentiation (27). Benzo(a)pyrene interferes with cell differentiation as a part of its carcinogenic mechanism (32). It may be speculated that the initial spurt in type III collagen synthesis may promote the various effects attributed to benzo(a)pyrene by altering the patterns of cell proliferation and differentiation. Collagen has been implicated in carcinogenesis and significant changes in the rates of collagen synthesis and in its nature (i.e., scar tissue formation), have been associated with carcinogenesis (5,6). Our limited studies support these ideas.

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R. BHATNAGAR: First of all I should apologize for the briefness of my abstract. I did not submit the full abstract, and planned on our discussion with personnel here and perhaps on the basis of a letter that I talked about - the kind of things that would be interesting. The abstract does not entirely reflect what I said here. I should also have named the coworkers that were involved in this. Our morphologist is a Dr. John Belton, a Professor of Biology at California State University at LaJolla Hayward, and presently is at Johns Hopkins University. Other coworkers are Dr. Muselam, Ms. Terento, and Dr. Si Duk Lee of the EPA have been involved in some of the agent studies. Now, as I discussed with Dr. Daniel, there has been quite a bit of interest in the role of collagen itself in carcinogenesis. Collagen is a mitogen. Collagen has been shown to be present at the site of all sorts of injuries, and it has been said many times that cancer commonly appears at the sites of old injuries. What I was tyring to imply here was that the type of collagen that appears in benzopyrene, treated cultures is a type that is associated with differentiating systems. A collagen that is associated with laboratory preparation of cells perhaps may have some significance.

Table III

RELATIVE SYNTHESIS OF TYPE I AND TYPE III COLLAGENS

IN LUNG ORGAN CULTURES EXPOSED TO BENZO(A)PYRENE

Culture Conditions	Percent Radioactivity Incorporated Into Collagen Chains Type I	Type III
Control	70	30
Benzo(a)pyrene	52	48

Cultures were pulse-labelled between 21 and 24 hours as described in Table II and analyzed for collagen chain composition.

General Discussion

F. DANIEL: Have you tried any other classes of carcinogens at this time?

R. BHATNAGER: No, these are preliminary studies and we have concentrated on benzopyrene and pyrene and we are planning to look at other chemicals. We have looked at cadmium incidentally and with cadmium we see the same kind of thing. I should point out, though, that these changes may have similarities to the process of carcinogenesis. We have heard many papers on the metabolism of polyaromatic hydrocarbons in which the metabolites eventually bind to DNA and transform it. If you have a system with such a process going on and there is a great increase in the poly factor within itself, then it is conceivable that the cells that have been transformed may somehow get amplified and we may see more of them and that many contribute to the process of carcinogenics.

J. VOSTAL: I would like to ask two questions. First of all you have been talking about the addition of the benzopyrene. Would you please tell us what the benzopyrene was dissolved in, and how did you mix it with the medium in which the tissue culture occurred? The second question is even much more important. If I am correct, in your abstract, you concluded that the changes you have seen may be related to carcinogenic alterations and may be used as early markers. Frankly speaking, we would like to know who is the morphologist who gave you the impression that you have seen any indication of the carcinogenic alterations?

APPLICATION OF A BATTERY OF SHORT TERM MUTAGENESIS AND CARCINOGENESIS BIOASSAYS TO THE EVALUATION OF SOLUBLE ORGANICS FROM DIESEL PARTICULATES

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ABSTRACT

The extractable organics from Diesel particulate emissions have been shown to be mutagenic in a bacterial screening assay (Ames, *S. typhimurium*). This report summarizes the results from a battery of confirmatory bioassays for mutagenic and carcinogenic activity. The test systems included in this battery are primarily mammalian cell systems, however, one assay was conducted in insects (*Drosophila*) and one in yeast (*Saccharomyces*). The bioassays detected the following biological effects: gene mutations, DNA damage, and oncogenic (neoplastic) transformation.

The Diesel particles, extracts, and fractions were generated from a Catepillar 3208, 4 stroke cycle, V8 engine used in urban service vehicles. The samples included a total dichloromethane extract (DCM) of the Diesel particles collected after dilution on a filter; a polar, strongly fluorescent fraction of the neutral organics (TRN) and the most polar fraction of the neutral organics (OXY).

The gene mutational assays in mammalian cells were positive with all samples tested including DCM, TRN, and OXY in the BALB/3T3 mouse cells and OXY in the L5178Y mouse lymphoma

cells. The X-linked recessive lethal assay in Drosophila melanogaster was negative for the OXY fraction administered by feeding 1 mg/ml for 3 days.

The bacterial DNA damage assays requiring either diffusion or killing were non-conclusive with these relatively non-toxic complex organics. The nitotic recombination assay in Saccharomyces cerevisiae D3 was positive with DCM and OXY and negative with the TRN fraction which caused no toxicity at the highest doses testable. Unscheduled DNA synthesis in WI-38 cells was negative with the OXY fraction.

Oncogenic transformation assays performed in BALB/3T3 Cl A31 mouse cells showed all three samples, DCM, OXY, and TRN to produced morphologically transformed colonies.

For further information, see Huisingh, J., et al., "Application of Bioassay to the Characterization of Diesel Particle Emissions" in Application of Short-Term Bioassays in the Fractionation and Analysis of Complex Environmental Mixtures (Environmental Science Research, Vol. 15), M. D. Waters et al., Eds., Plenum Press, New York, 1979, pp. 381-418.

General Discussion

J. VOSTAL: I am really very seriously concerned. I think you have mentioned that you have found some evidence of mutagenicity and potential carcinogenicity. You have been comparing the data on heavy duty motor vapors and light duty motor vapors and I still have the same problem that was raised yesterday, namely, why have only two revertents/microgram been shown in some of our tests while another one has been showing 200 of them. I think that we are living in the dirt of confusion, since we are always seeing on our slides the expression of a concentration which is given as a microgram per milliliter, but nobody states how it's possible to compare a microgram of the residium obtained after fractionation with a total extract and probably what [illegible] most important with the total mass of the diesel particles. I can hardly accept that anything could be really deduced from the data we have seen in this presentation the sample, which has been tested being obtained from two heavy-duty motor vapors, had about 50 or 25 percent of the extractable mass, and we are trying to apply it to a type of engine of the future producing a light duty vapor which barely has about five, or maximum eight percent of extractable material. We have already discussed it with EPA. I think it will be a great benefit if we abstain from using the data only showing micrograms per milliliter. We should really do the recalculation as an activity for microgram of the particulate. After that we should probably reassess our conclusions relating to dose-response. Practically all the tests which are being shown here as a documentation of the mutagenic effect are typically dose dependent, and it was very clearly demonstrated that you must accumulate a certain amount of micrograms before you show any effect. Obviously those micrograms have a completely different meaning. It could be a product of one heavy-duty engine as compared to the 50 light-duty motor vaports.

J. HUISINGH: All right, I think you have made some very good points. It is, of course, possible for us to calculate our data back that way and it does probably give a clearer idea related to the particle emissions. However, I think that at this point, particularly in this meeting, we would like to concentrate on testing for biological effects and in the process determines the best ways to test the materials, not to calculate revertents per mile and tumors per kilogram of fuel consumed, or that sort of thing. In the evening session we will present data from three light-duty vehicles; the Oldsmobile, the Nissan, and a Volkswagon Rabbit, as well as a heavy-duty vehicle compared to a gasoline vehicle - data that I think will help address that question. It does not appear that these vehicles which are light-duty vehicles are necessarily less active. In some cases they are considerably more active. There is a difference between the vehicles and that certainly is something that will make some interesting discussion. We have not and will not be presenting that data expressed per mile, but all of the data is there if you want to make those multiplications. I think we are just a little hesitant at this time, until we understand more about the systems, to express the data that way because I think it may encourage people to jump to conclusions. We are not looking at man exposed over a lifetime. These cells in some cases were exposed two hours. These mammalian cell assays were all corrected for toxicity, but after the two-hour exposure the cells were washed and were then plated out or cultured in the absence of the test substance. In the Salmonella data we have seen some different reported numbers, even when you correct the toxicity by the estimated method that Dr. Thilly described, that is an estimation of toxicity. The toxicity is not determined and handled exactly in the same way that is possible for the self culture assay. I think the range of Salmonella data you referred to, two to 200 revertents per microgram, is being confused with some fractionation work and total extracts, because if you look at the total extracts over the whole range of vehicles we have tested and other other people have tested, and was done in three separate laboratories, we never see that range of activity. We see very consistent numbers between vehicles run on different days when the samples are handled the same way.

A REVIEW OF IN-VITRO TESTING SYSTEMS APPLICABLE TO DIESEL HEALTH EFFECTS RESEARCH

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ABSTRACT

In-vitro mutagenicity and carcinogenicity testing techniques are currently being used to assess the potential risk to man of exposure to diesel exhaust emissions. This paper examines general considerations of such systems, the types of *in vitro* tests currently available, the advantages and disadvantages of each cell line and type of test, the limitations of *in vitro* techniques, the alternative human cell lines that could be utilized for diesel health effects studies, and recommendations for future research employing *in vitro* methods.

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1. INTRODUCTION

Diesel exhaust emissions contain numerous organic compounds, including polycyclic aromatic hydrocarbons (PAH) which may have a potential impact on human health (Santodonato, et al., 1978). Various PAH compounds, which are adsorbed as condensed material on particulate matter, are thought to be the major contributors to the mutagenic activity of organic extracts of diesel particulates (Briggs, et al., 1978). Adsorption of these compounds to particulates may significantly increase their carcinogenic effects (Katz & Pierce, 1976). Some of these PAH compounds, particularly the lipid-soluble neutral fraction and the basic fraction, have great affinity for lung tissues (Philpot, et.al., 1977).

In vitro tests have been developed over the last decade which can provide a rapid appraisal of the mutagenic/carcinogenic potential of various compounds or mixtures of compounds. Several agency and interagency groups have reviewed current in vitro test procedures (Science News, 1979). There are three general types of in vitro tests - biochemical studies, bacterial mutagenesis tests, and tests employing mammalian cell cultures:

- Biochemical testing examines adduct formation of the activated forms of the carcinogens with nucleophilic centers on biological macromolecules, e.g., protein, ribonucleic acid (RNA), deoxyribonucleic acid (DNA). The most useful information comes from looking at adduct formation with DNA since this is the center of interest in terms of genetic damage and carcinogenesis. Adduct formation with blood cell proteins has been suggested for diesel health effects work, although this is a very indirect measure and may not be a good index for the amount of reactive PAH metabolite that would bind to DNA in low concentration ranges (U.S. EPA, 1979). DNA repair studies also fall into this category.

- In vitro microbial tests are tests for mutagenicity, i.e., the capability of a compound or mixture of compounds to cause a mutation. The most widely used mutagenicity assay, and the one accepted by EPA, is the Ames test (Salmonella). Various assays employing cultures of other microbes supplemented with mammalian microsome preparations can also be used.

- In vitro mammalian cell culture studies include tests for mutagenicity and carcinogenicity performed in primary or continuous cell lines.

Researchers ultimately would like to extend the results of in vitro tests to man in terms of relating a given exposure to the risk of cancer. An individual's risk of developing lung cancer is dependent on three groups of "determinants". These are 1) exposure to carcinogens, 2) exposure to modifying factors in the environment (cocarcinogens, syncarcinogens, anticarcinogens), and 3) predisposing host factors. Most in vitro studies address the first issue, although the first and the second issues can also be addressed together in properly designed in vitro studies. Individual variation in susceptability, which is part of the third factor may be approachable in mammalian cell systems by using heterogeneous cell populations instead of cloned cell lines (Harris, et. al., 1978).

This paper provides a critical review of in vitro test systems that are being applied to or could be applied to studies of the health effects of diesel exhaust emissions. Section 2 presents general biochemical considerations to be kept in mind when comparing various cell systems used in in vitro tests. Section 3 provides an overview of in vitro mutagenesis, cell transformation, and biochemical tests currently available. Section 4 examines the limitations of these tests. Conclusions and recommendations comprise Section 5.

2. IN VITRO SYSTEMS - GENERAL CONSIDERATIONS

The final impact of a given PAH exposure to a particular cell ultimately depends on the rate at which metabolites are produced in a given tissue. These kinetics reflect both the number of molecules of activating enzyme present in the cell as well as the specific activity of the enzyme, expressed as nanomoles of PAH activated per mg of enzyme per minute. The higher the rate of conversion of PAH the higher is the effective dose of PAH metabolite to the target DNA. This rate may determine if mutagenesis or cell transformation occurs in an in vitro system and whether the lag time necessary to see the effect will be within the time allowed for scoring the test results. A compound that is detected as a mutagen or potential carcinogen by one in-vitro system may be missed by another in vitro system due to inherent differences in activating enzyme activities.

The major enzyme responsible for activation of certain PAH to mutagenic and/or carcinogenic forms is aryl hydrocarbon hydroxylase, which is part of the complex of oxidative enzymes referred to as P-450. It is thought that two different pools of AHH exist in any tissue at any given time. One represents constituent enzyme which is always present at some minimum level. The second represents an inducible enzyme pool whose level can be highly variable. Certain PAH can induce enzyme levels up to almost 10-fold depending on the species, strain, or tissue from which the cell line is derived (Philpot, et.al., 1977). Pulmonary AHH is highly inducible in cell lines derived from rat, hamster, and some strains of mice. The strain dependence of this characteristic is very strong in some animals such as mice, where the level of AHH induction is also dependent on the inducer compound used (Philpot, et.al., 1977). Some mouse strains, such as B6 and B6D2 are highly inducible. Others such as D2 and the first generation obtained from crossing C3H and D2 demonstrate little or no AHH induction (Wang, et al., 1976). Data for smokers versus nonsmokers suggest that pulmonary AHH activity can be induced more than 10-fold in man (Philpot, et. al., 1977).

Existing evidence confirms that the concentration and specific activity of aryl hydrocarbon hydroxylase (AHH) in a given tissue vary by an order of magnitude or more from species to species (Philpot, et.al., 1977). Furthermore, the metabolite pattern obtained in terms of relative proportions of specific metabolites also varies from species to species (Pelkonen, 1976). There are also differences in AHH activities between sexes and between tissues, the differences in the latter case being greater (Philpot, et. al., 1977).

There are also significant developmental differences in AHH activity. Cell lines derived from fetal tissue show the same tissue variability in PAH metabolism as cell lines derived from adult tissues. Fetal liver cells have much greater (about 2-fold) induced AHH activity than fibroblasts derived from fetal lungs or skin (Pelkonen, 1976), although uninduced activities for conversion of benzo(a)pyrene (B[a]P), dimethylbenzanthracene (DMBA) and N-2-fluorenylacetamide (FAA) are about the same (Juchau, et. al., 1978).

In humans and presumably in animals there is also significant individual variation in AHH activity (Paigan, et.al., 1978a). Many highly variable factors including developmental, genetic, dietary, hormonal/ physiological, disease, and behavioral (e.g., smoking) factors may be correlated with levels of AHH (Boulos, 1978). Human liver AHH levels vary up to 16-fold and may be strongly influenced by smoking and general health (Pelkonen, 1976). There is some evidence supporting the theory that those persons with high AHH inducibility and activity are at higher risk of lung cancer compared to individuals with lower activities or inducibilities (Paigan, et.al., 1978); Busbee, et.al., 1979). AHH levels in cultured human lymphocytes and pulmonary alveolar macrophages from cancer patients support this correlation (Busbee, et.al., 1979).

Generally, metabolic activation is obligatory for the expression of the mutagenic/carcinogenic potential of PAH (Nagao and Sugimura, 1978). Many in vitro mutagenesis and cell transformation assays are supplemented with microsomes preparations from a mammalian species (usually rat liver) or with another cell type to aid in conversion of PAH to their active forms. In the case of microsomes, the animal from which they are obtained is usually treated with a compound that induces P-450/AHH to higher than consituent levels. Routinely, Arochlor has been used, although this may not be a wise choice due to its toxic and carcinogenic nature which may cause undesirable interactions with the test material. Some PAH such as 3-methylcholanthrene (3-MCA) and B(a)P which are also used for this purpose in some systems have the same inherent problems. If this problem is to be circumvented, these compounds are easily replaced with a mixture of phenobarbitol and 5,6-benzoflavone, which is as equally as potent an inducer as 3-MCA or B(a)P (Nagao and Sugimura, 1978). In some instances, however, such interactions as co-mutagenesis, co-carcinogenesis, and syncarcinogenesis are desirable in an in-vitro system as they increase significantly the sensitivity of the system for detecting mutagens and carcinogens.

Ideally, activation kinetics of the in vitro systems applied to diesel particulate extracts should reflect that seen in lung tissues, preferably human lung. This suggest adaptions for certain currently-used protocols. For example, in microbial (bacteria or yeast) mutagenic systems the conventionally used rat liver microsomes could be replaced with human lung microsomes, when available, to better represent the lung metabolic processes, both qualitatively and quantitatively, that occur in man.

3. TYPES OF IN VITRO TEST SYSTEMS

3.1 MUTAGENESIS

In vitro mutagenesis assays include microbial (bacteria, yeast) and mammalian cell systems. In the forefront of the microbial categoy is the Ames test which uses various histidine-requiring mutants of the bacteria *Salmonella typhimuium*. Mutation is detected in the Ames test as conversion of individual cells to a histidine-independent state. These mutants include TA 1537 and TA 1538 which are frame-shift mutants, TA 1535 which are base-pair substitution mutants, and TA 100/TA98 which are derived from TA 1535 and TA 1538. The relative sensitivity of these various strains in detecting mutagens/carcinogens varies as a function of the compound tested (Juchau, et. al., 1978; Nagao and Sugimura, 1978).

Another type of bacterial test system involves DNA repair as a parameter of damage in DNA polymerase-deficient mutants (pol A_1) of *E coli*. This test is based on inhibition of growth of pol A_1 mutants by compounds that alter DNA. Microsomal activation is required for PAH studies using this sytem (Nagao and Sugimura, 1978).

The third type of bacterial system involves prophage (bacterial virus) induction in *E. coli*. Specifically one can use an *E. coli* mutant (envA, uvrB) which produces a defective membrane (allowing facile penetration of PAH) and which is deficient in DNA repair. Both mutations result in high sensitivity to PAH. However, not all PAH have phage inducing capability. Again microsomal activation of PAH is required (Nagao and Sugimura, 1978).

Mammalian cell systems are also used for detecting mutagens and comparing mutagenicity of various substances. The cell types employed for mutagenicity studies can be divided into two categories: permanent cell lines which have been in culture for several generations of cells, and cells derived directly from fresh tissue (primary cell cultures). The cell strains and genetic markers employed are listed in Table 1. The fact that the PAH's need to be metabolized to "proximate" or "ultimate" forms capable of binding to DNA was not recognized during early efforts of mutagenesis testing with the pure compounds. Thus, many of the pre-1970 PAH mutagenicity studies in both bacteria and mammalian

TABLE 1. TARGET CELL TYPES AND GENETIC MARKERS EMPLOYED FOR INVESTIGATING MUTAGENESIS IN MAMMALIAN CELLS BY PAH'S AND DERIVATIVES*

Investigators using established cell lines (aneuploid, high plating efficiency, infinite lifespan)	Investigators using diploid cell strains (diploid, relatively lower plating efficiency, finite lifespan
1. Chinese hamster cells	1. Syrian (golden) hamster embryo cells
a. V79 hamster lung cells 1) Brookes and colleagues; AG^r	a. Barret, Ts'o and colleagues; OUA^r
2) Huberman and colleagues; AG^r, OUA^r	b. Huberman et al., OUA^r
3) Krahn and Heidelberger; AG^r	
4) Malaveille et al.; AG^r OUA^r	2. Human skin fibroblasts: normal and repair deficient
5) Marquardt et al.; AG^r	
6) Wood, Wislocki and colleagues; AG^r	Maher, McCormick et al.; AG^r
b. CHO hamster ovary cells 1) Hsie et al.; TG^r 2) Huberman and Sachs; ts reversion	
2. Mouse hymphoma cells L5178Y Clive; $BUdR^r$, TG^r	

SOURCE: Maher and McCormick, 1978.

* Abbreviations: AG^r = 8-azaguanine resistance; OUA^r = ouabain resistance; TG^r = 6-thioguanine resistance; ts = temperature sensitivity; $BUdR^r$ = bromodeoxyuridine resistance.

cells did not provide metabolic activation systems and yielded negative results. Methods used to provide metabolic activation for mammalian mutagenesic detection systems are outlined in Table 2. For cell lines that do not have sufficient P-450 activity, activation can be provided by a microsome preparation or a "feeder" layer of a different cell type which does have PAH metabolic capability. An example of the latter is the use of Syrian hamster embryo (SHE) primary cell cultures as a feeder layer in a Chinese hamster ovary (CHO) cell system. This particular system has some advantages in that it can yield higher activities (higher mutation frequencies) than microsomal preparations and does not have the problem of nonlinear activation responses at moderate to high S-9 concentrations (above 0.5 mg/ml). This type of system also has less variability than a microsomally-activated assay (Carver and Felton, 1979).

TABLE 2. METHODS USED TO PROVIDE REACTIVE METABOLITES OF PAH'S FOR ASSESSING MUTAGENICITY IN MAMMALIAN CELLS

1. Chemical or biosynthesis: reactive metabolite or derivative prepared chemically or isolated following enzymatic synthesis

2. Microsomal-mediated: production of reactive metabolites from parent or intermediate compound by cell homogenate fraction not of target cell origin

3. Cell-mediated: production of reactive metabolites by feeder layer present with target cells

4. Host-mediated: metabolites formed in body of host animal in presence of target cells

5. Metabolism within target cell itself: target cell has sufficient PAH-metabolizing capability

SOURCE: Maher and McCormick, 1978.

The potency of PAH varies in such mammalian mutagenesis systems over two orders of magnitude or more. Tables 3 and 4 show the relative mutagenic potential of various PAH compounds in a CHO system that utilizes lethally-irradiated rodent cells for metabolic activation (Huberman, 1978).

Some mammalian cells are more sensitive indicators of mutagenesis than bacteria (Kuroki, 1978). Furthermore, there are significant differences in mutagenicity between the bacterial and mammalian test systems in terms of magnitude of response to B(a)P metabolites. The ability to induce mutations in V79 cells appears to be more closely related to carcinogenicity than induction of revertants in Salmonella (Kuroki, 1978).

TABLE 3. INDUCTION OF OUABAIN-RESISTANT MUTANTS IN THE CELL-MEDIATED ASSAY BY DIFFERENT CARCINOGENIC HYDROCARBONS

Hydrocarbon	Concentration of Hydrocarbon (μg/ml)	Number of Ouabain-resistant Mutants per 10^6 survivors
Control	0	1
Benzo(e)pyrene	1	1
Phenanthrene	1	1
Pyrene	1	1
Benz(a)anthracene	1	2
Chrysene	1	2
Dibenz(a,c)anthracene	1	3
Dibenz(a,h)anthracene	1	4
7-Methylbenz(a)anthracene	1	24
3-Methylcholanthrene	1	108
Benzo(a)pyrene	1	121

SOURCE: Huberman, 1978.

TABLE 4. MUTABILITY OF DIFFERENT GENETIC LOCI IN THE CELL-MEDIATED ASSAY BY CARCINOGENIC HYDROCARBONS*

	Number of mutants per 10^6 cells		
Hydrocarbon	Temperature-Resistant	Ouabain-Resistant	8-Azaguanine Resistant
Control	0.6	1	60
Pyrene	0.9	1	50
Phenanthrene	0.7	1	80
3-Methylcholanthrene	125	108	3660
Benzo(a)pyrene	170	121	4250

*Cells were treated with 1 ug/ml of the polycyclic hydrocarbons.

SOURCE: Huberman, 1978.

Induction of sister chromatid exchange (SCE) in mammalian cell cultures represents another in vitro mutagenisis system. V79 cultured cells have been used for this purpose. The SCE assay is a rapid and sensitive method for evaluating the interactions of chemicals with DNA. This assay has been used for various PAH and environmental samples including cigarette smoke condensate (Lockard, et.al., 1979).

3.2 TRANSFORMATION

Numerous cell lines and protocols exist for in vitro study of chemical induction of neoplastic transformation. These systems involve primarily two cell types - fibroblastic or epithelial cells.

Fibroblasts are the best-characterized and perhaps the easiest to grow, and have been employed for much if not most of the basic research into the mechanism of cell transformation. One major advantage in using fibroblasts is that, unlike epithelial cells, chemical carcinogens induce drastic morphological changes in fibroblasts which are easily detected and quantitated. Such quantitative indices of transformation of epithelial cells are lacking, and thus the study of these cells is more difficult. Secondly, fibroblasts have a much shorter response time than epithelial cells. Neoplastic transformation in fibroblasts can be detected in 1-4 weeks, whereas in epithelial cells this lag time is measured in months.

Currently, the cells widely-used for PAH transformation studies include the SHE cells and the various mouse lines. SHE cells exhibit morphological changes in about 1 week, the extent of which is dose dependent. The major advantages of SHE are that its chromosomes are similar in number and morphology to human cells, that methodologies involving this cell line are well developed, and that these cells have a low spontaneous transformation rate. The disadvantages of SHE cells are that growth is slow in early passage numbers and that a mixed population of cells is used (although the cells becomes more homogeneous as they divide and grow).

Another type of system uses aneuploid mouse cell lines including C3H mouse embryo cells, their cloned lines such as C3H/10T½, Balb C mouse embryo cells and 3T3, which are derived from Balb C. These cell lines are contact inhibited, grow in a uniform monolayer, and form foci of transformed colonies which "pile up" upon treatment with a carcinogen. Time for obtaining results from these cell lines is usually four to six weeks.

The major advantage of these cells is that they are cloned populations derived from a single cell and, therefore, are genetically homogeneous. Like SHE cells, they display variations in transformation responses depending on culture conditions, particularly the concentration and characteristics of the serum used.

There are several different indicators of cell transformation, and, therefore, clarification is needed to delineate which characteristic or property is being transformed (e.g., *neoplastic* transformation, *morphological* transformation). Some of these indices are listed in Table 5. Not all of the transformations in properties can be associated with production of an abnormal tumorigenic cell. Some changes represent just one step in the sequence of events leading to truely neoplastic cells. For example, enhanced fibrinolytic activity and morphological transformation of the cells are noted soon after treatment with a carcinogen. However, growth in soft agar, which is truly characteristic of a

TABLE 5. PHENOTYPIC TRANSFORMATIONS OF NEOPLASTIC CELLS

Phenotypes

1. Tumorigenictity
2. Loss of anchorage dependency for growth
    a. Soft agar/agarose
    b. Methyl cellulose
    c. Teflon
3. Morphological changes
4. Enhanced fibrinolytic activity
5. Chromosomal changes
6. Indefinite life span
7. Lack of density-dependent inhibition of replication
8. Growth at low serum concentrations
9. Changes in membrane properties
    a. Altered glycoproteins and glycolipids
    b. Increased rate of transport
    c. Loss of high molecular weight, surface glycoprotein
    d. Increased agglutinability by plant lectins
    e. Increased mobility of membrane proteins
    f. Altered surface structure (SEM)
10. Changes in microtubules and actin cables
11. Decreased cAMP levels.

SOURCE: Barnett and Ts'o, 1978.

malignant cell, is not noticed until after some 32-75 cell divisions. Some of these indicators need weeks to develop and may be unobserved due to use of premature analysis time in the test protocol (Barrett and Ts'o, 1978).

3.3 ADDUCT FORMATION AND DNA REPAIR

One can initially detect DNA damage by looking at DNA repair activities within the affected cell, or at DNA adduct formation with the active metabolites of the mutagens/carcinogens. Unscheduled DNA synthesis (DNA repair) involves excision of some of the damage caused to DNA, and synthesis of new segments of DNA. A large amount of DNA damage induces high levels of DNA repair activity, but not all the damage may be repaired. DNA-repair deficient cell strains are especially sensitive to mutagenesis and transformation (McCormick and Maher, 1978). Thus, cell transformation can be related to unscheduled DNA synthesis.

To determine the extent of DNA adduct formation, one can examine either RNA or DNA binding of radio-labelled PAH or metabolites, e.g., ^{3}H-B(a)P. DNA adduct determinations must be made soon after treatment of the cells with the test compound, since the proportional composition of the adducts changes with length of time after treatment. For B(a)P, and presumably other PAH, adduct formation is quite a sensitive method for some cell lines, e.g., hamster embryo cells (Baird and Dumaswala, 1979).

3.4 ALTERNATIVE HUMAN SYSTEMS

For studies of PAH or complex mixtures of PAH, as in the case of diesel exhaust particulate extracts, use of *in vitro* systems employing human cell lines would be especially relevant, two human cell systems suggest themselves - skin and lung. Human foreskin cells are readily available and do well under culture conditions. This system has already been applied to the study of cytoplasmic PAH binding protein activity and its role in transporting PAH to the nucleus of the affected cell (Tejwani and Milo, 1979). Suitable lung cells can come from lung tissue itself or from the mobile cells within the lung, e.g., the pulmonary alveolar macrophages (PAM's) and lymphocytes. Some studies have successfully utilized mixed populations of both PAMS and lymphocytes in studying the activation and detoxification of PAH compounds (Marshall, et.al., 1979).

4. LIMITATIONS OF TESTS

There are several limitations to *in vitro* tests which place constraints on interpretation of results obtained with them. Some of these limitations are due to problems inherent in the test systems themselves. Additionally, results with *in vitro* systems for detecting mutagenesis/carcinogenesis represent only rough indications of what may happen *in vivo* (in the whole animal) due to several irreconcilable differences between isolated cells and the intact organism, such as:

- Differences in hormonal levels, which affect general overall metabolic activity and rates.
- Differences in thresholds, if any exist, for mutagenic/ carcinogenic effects.
- Differences in relevant pharmokinetic factors such as rate of absorption, effective dose delivered or experienced by the tissue, rates of activation and detoxification.

The most widely used mutagenicity assay, and the one accepted by EPA, is the Ames test (Salmonella). There is some controversy as to whether the Ames assay can be used as a predictor of carcinogenesis. There are opinions and evidence pro (Ames and Hooper, 1978) and con (Ashby and Styles, 1978; DeFlora, 1978; Maugh, 1978a). The fact that the Salmonella assay can yield both false positive and false negative results (the latter being of greater consequence) is the major restriction of this technique.

Furthermore, due to biochemical differences between animals of various species, activation of test compounds by microsomes isolated from one species may yield a positive Ames test result, while carcinogenicity testing in a different species may yield negative results (Ashby & Styles, 1978a). In addition, 14 variables have been identified which influence the numerical results obtained with the Ames test (Ashby and Styles, 1978b). In particular there is great variability between liver microsome preparations and thus, they should be standardized for carcinogen-activating and carcinogen-deactivating enzymes. For example, differences in how liver microsomes are prepared can result in a variation by more than a factor of 100 in the observed mutagenic potency of benzo(a)pyrene (Maugh, 1978a). Critics say that no correlation should be drawn between Ames test results and carcinogenic potential, especially for PAH, nitrosamines, polycholorinated cyclic compounds, aza-napthols, symmetrical hydrazines and steroids, which yield a high proportion of false negatives (Maugh, 1978b).

Mammalian mutagenesis systems also have their limitations. Mammalian systems lack reproducibility for quantitative determinations due to such things as sensitivity to variations in initial plating density and insufficient incubation time for expression of all mutations. The latter problem is due to the fact that PAH and PAH metabolites in mammalian systems exhibit dose-dependent delay of the response. That is, the smaller the dose, the longer is the lag period before mutation is expressed. This is presumably partly due to low metabolic rates for PAH in some cell lines. This problem can be alleviated somewhat by choosing a cell line that has inherently high rates of activation of PAH (Maker and McCormick, 1978).

Low metabolic rates also may be responsible for the fact that in some mammalian mutagenesis assays, the frequency of mutation is not proportional to dose at high doses. That is, high doses of PAH fail to give the number (frequency) of mutants expected on the basis of responses at lower doses. This may be due to saturation of the AHH activation systems in some cell lines (Maher and McCormick, 1978).

There are several things which affect cell transformation results. These include cell density, fixation of DNA damage, and factors in the growth media. The relationship to cell density is that cell cultures in confluency (fully-grown monolayer within the physical constraints of the culture plate) have been found to be relatively resistant to transformation by polycyclic hydrocarbons compared to that seen in growing cultures. This is true for mouse cell lines and some hamster cell lines such as SHE. This is most likely connected with the need for cells to "fix" DNA damage via several cell divisions before transformation can be expressed. Experiments with mouse 3T3 cloned cells indicate that about 4 cell divisions (generations) are required to fix the damage and result in expression. The transformation frequency (the number of transformed foci per number of cells) decreases with increases in initial cell density, with increases in cell density at time of treatment, and with decreases in number of cell generations required to attain saturation density (confluency). Additionally, serum factors consumed by the cells are related to inducibility of AHH. If cells are depleted of serum factors, e.g., as in the confluent state, then there is low AHH activity and subsequently low sensitivity to PAH. Serum factor depletion may tie in with the cell division requirement mentioned above in that serum factors are required for normal cell growth. This is significant for testing both in terms of qualitative and quantitative results (Kakunaga, 1978).

5. CONCLUSIONS AND RECOMMENDATIONS

There are two perspectives from which an in vitro system may be judged appropriate for diesel health effects research. One is on the basis of sensitivity of the system to mutagenesis or carcinogenesis by PAH or PAH metabolites. The other is on the basis of biochemical and physiological similarily to the human tissues that would be exposed to and affected by diesel exhaust emissions.

Two things that can be done to enhance the sensitivity of a mutagenicity/carcinogenicity assay is to account for interferences that reduce mutagen/carcinogen detection potential (e.g., anticarcinogens in the growth media) and to screen candidate cell lines for AHH activity and inducibility. Rapid and simplified methods exist for doing the latter (Miyazawa, et al., 1977).

For more meaningful comparisons of data between laboratories and within laboratories, in vitro mutagenicity and carcinogenicity test protocols should be standarized. This should include:

- the confirmation of dose-response patterns
- the use of a standard latent period between the time of treatment with the test sample and the scoring of results for a given cell line
- the use of a standardized media for a given cell line
- the confirmation of cytotoxicity or cell viability under test conditions, provision of both activated and nonactivated test systems where appropriate, and provision of both positive and negative controls
- the use of a battery of in vitro tests to fully and accurately screen fractions from diesel particulate extracts.

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THE DNA DAMAGE ACTIVITY (DDA) ASSAY AND ITS APPLICATION TO RIVER WATERS AND DIESEL EXHAUSTS

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ABSTRACT

An extremely sensitive assay has been developed for DNA-damaging chemicals using a DNA repair-deficient strain of *Escherichia coli*. Considerable DNA-damaging activity has been demonstrated by this assay in natural surface-water samples and in diesel-exhaust particulate extracts.

INTRODUCTION

All common biological assays (such as the Ames technique) for traces of potentially mutagenic and carcinogenic chemicals require concentration ranges of mutagenic chemicals far above those encountered in the environment. It is therefore necessary to collect relatively large amounts of material and to extract and concentrate the mutagens by elaborate procedures. This severely limits the use of these systems for examining the environment.

We therefore set out to develop an effective bioassay for screening surface waters and other environmental materials. Such an assay would have to be sensitive enough to detect potentially mutagenic chemicals at environmental concentration ranges. It must also be simple and rapid, so that large numbers of samples could be examined with minimum effort.

As one possibility we turned to the assay for DNA-damaging chemicals developed by Nishioka and Nabori (1,2). This assay measures the effect of such chemicals on the increase in turbidity of cultures of repair-deficient bacteria during a 4-h period. With our modifications this technique can detect DNA-damaging chemicals with much greater sensitivity. We then developed a standard assay which allows detection of extremely small amounts of potentially mutagenic chemicals. Because of the extreme sensitivity of the assay, environmental samples, such as surface water, can be examined directly.

In this report we describe the assay and its application to the examination of diesel-exhaust particulate extracts. These extracts were prepared in the Division of Air of the New York State Department of Environmental Conservation (DEC) under a project sponsored by the U.S. Environmental Protection Agency (EPA; Grant R805934010; Dr. Richard E. Gibbs, Project Director).

SPECIAL ASSAY METHODS

Strains. *Escherichia coli* strains WP2 *trp*, WP2$_s$ *trp uvrA*, WP10 *trp recA* and WP100 *trp uvrA recA* were obtained from Dr. Evelyn Witkin, Rutgers University, New Brunswick, NJ.

Glassware and other materials. Because of the sensitivity of the assay it is very important that all materials used be pure or clean and thus free of DNA-damaging chemicals. While well-cleaned Pyrex glassware may be used for preliminary growth of the culture, special glassware free of chemicals which damage DNA must be used for any direct contact with the assay medium. Otherwise the growth of WP100 will be inhibited, ruining the assay. In particular, any materials sterilized with ethylene oxide gas or other chemical sterilizing agents must be avoided.

We have had little success reusing any type of glassware, especially Pyrex. For that reason and for convenience we used disposable glassware. Plain flint, 3-oz. prescription bottles (Brockway) were "tissue-culture" washed (i.e., without detergent), rinsed for 30 s with distilled deionized water, and sterilized in an electric oven at 350°C for 2 h. For pipetting nonsterilized 1-ml Biotips (Schwartz/Mann) were used. Commercially available 0.1- or 0.2-ml Biotips cannot be used, since they have been chemically sterilized. Special 0.1-0.2-ml Biotips, tissue-culture washed but not chemically sterilized, were prepared for us by Schwartz/Mann Division of Becton Dickinson Co., Orangeburg, N.Y. We had little success using Pyrex glass pipettes, although Thermex glass tuberculin syringes (no tips) could be used once but not reused.

Culture growth. Cultures were grown overnight and kept frozen at -73°C in 0.3-ml of minimal broth plus 10% dimethyl sulfoxide (DMSO) in 1-dram vials. The day before the assay the contents of one vial was added to 20 ml of minimal medium plus L-tryptophan and incubated for 6-7 h at 37°C on a reciprocating shaker. The minimal medium used routinely was Davis minimal broth (Difco) supplemented with 20 μg of L-tryptophan/ml and 1% glucose (MBD). Distilled deionized water was used to prepare all media.

After this incubation 0.1 ml of the culture was added to 20 ml of MBD (tryptophan, 40 μg/ml) and grown overnight (4 p.m.-7 a.m.). The culture was then adjusted with MBD at 37°C to an absorbance of 0.5 at 660 nm, using a Zeiss PMQII spectrophotometer, and was incubated with shaking for about 90 min or until the absorbance reached about 0.8. (If this absorbance was passed, the culture was adjusted back to 0.8 with MBD at 37°C).

This culture must be used immediately as the inoculum for the assay, which was usually set up while this culture was growing.

Assay procedure. With all weighable materials including reference materials (mutagens) 1 mg was weighed out and dissolved in 20 ml of DMSO or other solvent (50 μg/ml). Appropriate serial dilutions (usually 1 ml into 9 ml of MBD) were then made using prescription bottles. This was done immediately before the experiment, since many mutagens in solution are unstable at low concentrations. All subsequent operations were carried out in a 37°C walk-in incubator, where prescription bottles and all growth media had been stored the night before to ensure temperature equilibration.

Aliquots (0.1 ml) of serial dilutions of the suspected DNA-damaging chemicals were added to the bottom of these prescription bottles in an upright position. Appropriate controls were prepared using serial dilutions of the solvent (usually DMSO). A suspension of the inoculation culture was made (1 ml per each 49 ml of MBD at 37°C) and mixed well. A prewarmed Cornwall pipetter set at 4.9 ml was used to rapidly dispense this culture into the prescription bottles with and without chemicals and into empty bottles. (The highest final concentration of the weighed material was 1 µg/ml of growth medium.) This addition must be very rapid (bottle caps were removed beforehand) to ensure an accurate zero time point. In general 100 bottles could be inoculated within 2 min. At this point a timer was started, and the bottles were shaken gently and put on their sides for incubation. We did not use aeration, since many mutagens are apparently sensitive to oxidation, which reduces the sensitivity of the assay. All samples were duplicated.

Control bottles containing only the culture suspension were checked periodically for turbidity increase to follow growth, until an A_{660} of 0.18 was reached. All bottles were then rapidly placed in an ice bath to halt growth. Bottles were then selected at random and warmed rapidly to room temperature. Their contents were mixed with a vortex mixer and their A_{660} determined, using matched 3-ml-square glass cuvettes (1-cm light path) in a Zeiss PMQ II spectrophotometer. MBD at room temperature was the blank. Figs. 1 and 2 show further details.

Interpretation of results. The assay relies primarily on *E. coli* strain B/r WP100 *recA uvrA*. Because of genetic mutations at two loci this strain lacks the enzymatic capacity to carry out the two major pathways for repair of DNA after environmental damage. These pathways are the excision-repair system (uvrA+) and the postreplication recombination repair system (recA+). Thus even very slight DNA damage, such as that exerted by very low levels of mutagenic chemicals, is presumably nonrepairable. The failure in DNA replication and consequently in cell division and growth are measured as an inhibition of the expected increase in turbidity. Since almost all repairable DNA damage has a mutagenic effect, a DNA damage assay based on this organism is a valid measurement of potential mutagenicity.

Any difference in the effects on WP100 and on the repair-proficient strain (WP2) confirms the genetic toxicity of a given compound or sample. Both strains are routinely tested simultaneously; the effect on WP2 is usually very low. In addition, the recA (WP10) and uvrA (WP2s) strains can be used to differentiate toxic effects due to failure of excision repair as opposed to postreplication repair, when this is of interest.

RESULTS AND EXPLANATION

The effects of three direct-acting DNA-damaging chemicals are demonstrated in Fig. 1. Aroclor 1254, a polychlorinated biphenyl mixture, at three concentrations (10^{-2}, 10^{-6} and 10^{-8} μg/ml) inhibited growth for some 70 min; recovery then occurred rapidly. MNNG at the same concentrations slowed growth but did not completely prevent it. After about 120 min rapid growth occurred. These data suggest a pause in growth after turbidity has doubled in the presence of MNNG. The effect of mitomycin-C was similar to that of MNNG, although the increase was slowed somewhat more effectively at first.

We believe that the increased growth during subsequent incubation reflects the action of a residual, inducible repair mechanism in WP100, which can repair low-level chemical damage to DNA. Peters and Jagger (3) have demonstrated repair of near-UV lethal damage in an organism of this genotype by an inducible recA+ gene-independent system. Nishioka and Nabori's 4-h assay would allow time for such recovery to occur and would therefore be less sensitive to DNA-damaging chemicals.

In developing our DNA damage activity (DDA) assay, we prevented such recovery by reducing the incubation time. It is evident from Fig. 1 that when the chemical-free control culture has reached an A_{660} of 0.18, the assay has achieved maximum sensitivity in measuring the inhibition of turbidometric increase. For that reason, in our standard assay we stop the incubation at that point.

A typical assay for MNNG is shown in Fig. 2. It is evident that the recA+ gene product can also repair the DNA damage involved, since the WT and uvrA strains are much less sensitive to MNNG.

The combination of the recA and uvrA genes in WP100 increases the sensitivity considerably over recA.

The recA gene renders *E. coli* more sensitive to most DNA-damaging chemicals; and the DDA assay is sensitive to a variety of such chemicals (Table 1). Benzo(α)pyrene, which requires metabolic activation in the Ames assay for mutagenicity, is apparently detectable without activation in the DDA assay, although its low level of activity could be due to a contaminant. The DDA assay also detects polychlorinated biphenyls (PCBs), which are not mutagenic in the Ames system. Since PCBs are a common contaminant in surface water, this sensitivity increases the assay's potential value in studies of environmental water contamination, although perhaps limiting its value as a specific indicator of mutagens.

APPLICATIONS

River water. To evaluate the utility of the DDA assay in examining the environment, we measured DNA damage activity in river samples from two river systems in the northern United States (Fig. 3). The results indicated measurable contamination in the Mohawk-Hudson river system in the Schenectady-Troy-Albany urban area and downstream. Concentrations of DNA-damaging chemicals in the Buffalo-Niagara river systems in the Buffalo metropolitan area are several orders of magnitude higher. Assessment of the ecologic and public health significance of such contamination requires further study, especially to

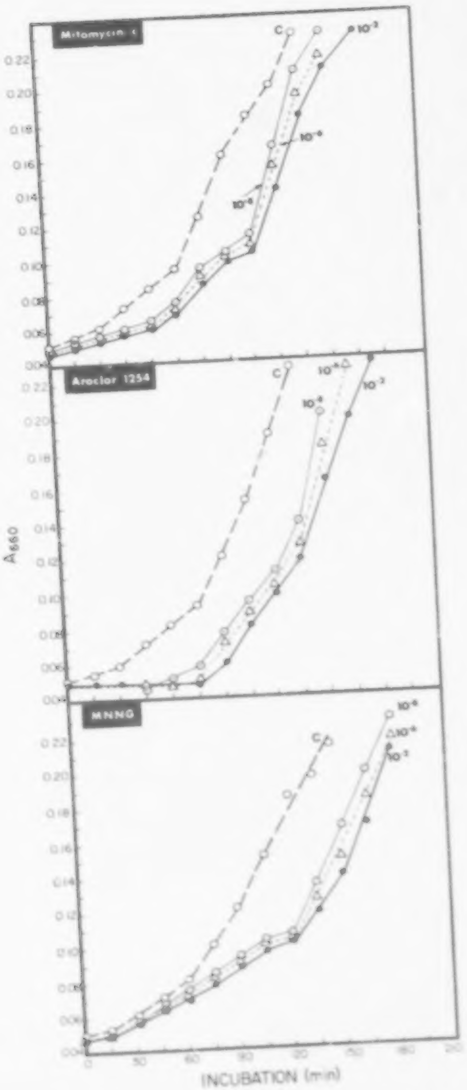

Figure 1. Effect of three concentrations (μg/ml) of Aroclor 1254 (Monsanto Company), N-methyl-N'-nitro-N-nitroso-guanidine (MNNG) (Aldrich Chemical Company) and mitomycin-C (Calbiochem-Behring Corp.) on turbidity increase in _E_. _coli_ WP100 _trp_ _recA_ _uvrA_.

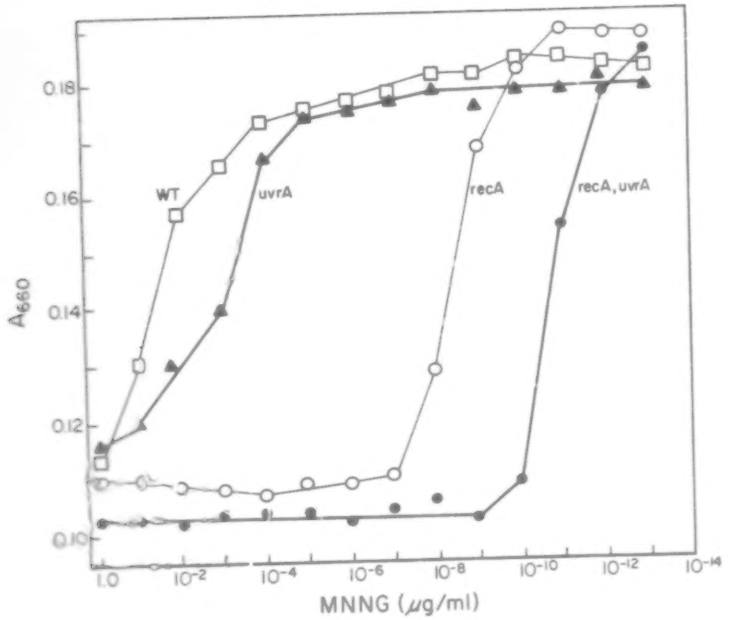

Figure 2. Standard DDA assay of MNNG. The diluted cultures of the four strains are grown with a range of concentrations of the suspected DNA-damaging chemical until the control culture (containing no mutagen) reaches an A_{660} of about 0.18. In carrying out this operation, one bottle is used for each reading of turbidity. We take duplicate readings every 10 m or so as the cultures approach an A_{660} of 0.18 and then read the turbidity in 6 or more bottles to verify the final reading at 0.18. The effects of the various concentrations of the chemical on turbidity increase are then compared. The results may be quantitated by projecting the point of 50% change between the base level and the final level of turbidity to the axis.

TABLE 1. ACTIVITY OF SELECTED MUTAGENS IN THE DDA ASSAY WITH _E_. _coli_ _recA_ _uvrA_

Chemical	Minimum detected concentration (μg/ml)*
MNNG	10^{-10}
4-nitroquinoline	10^{-8}
Mitomycin-C	10^{-10}
Ethylmethansulfonate	10^{-7}
2-Nitrofluorine	10^{-10}
Benzo(α)pyrene	10^{-5}
Sodium bisulfite	10^{-9}
Aroclor 1254 (PCB mix.)	10^{-10}

* Lowest concentration giving maximum inhibition in the DDA assay, performed as in Fig. 2. Results with the other three _E_. _coli_ strains were comparable to those reported for MNNG in Fig. 2.

determine whether the particular DNA-damaging material is known or suspected to be hazardous to humans.

This assay system should provide a rapid, low-cost, and extremely sensitive method of surveillance of contamination of surface water by DNA-damaging chemicals. (Many sources of public water supply are surface water.) By working upstream from contaminated areas, sources of contamination could be pinpointed. By frequent sampling at selected points, sudden releases or spills of suspect materials could be detected. Correlation of results over an extended period with ecologic and public health data could indicate biologic effects of chronic contamination of streams with DNA-damaging material.

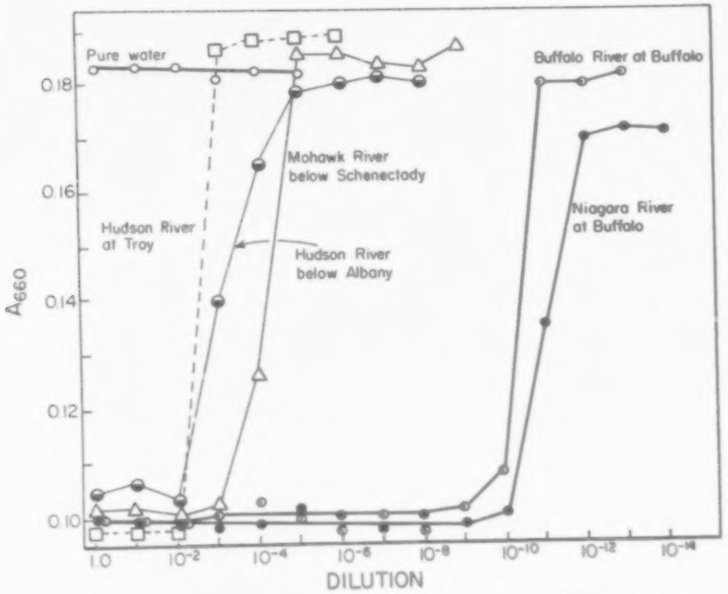

Figure 3. DNA-damaging activity of river water from two river systems, the Mohawk-Hudson near Albany and the Niagara-Buffalo near Buffalo. Only results with the *recA uvrA* strain (WP100) are reported, since the other three strains gave much lower responses. Tenfold serial dilutions into MBD were made from a mixture of 9 ml of undiluted filter-sterilized water (kept on ice after collection until use) and 1 ml of 10X concentrated MBD. The pure water control was distilled-deionized water commonly used in the laboratory. The river water was collected by dipping at the surface, and was transferred into prescription bottles or 100 ml glass-stoppered bottles. Control pure water held in these bottles during storage of the samples showed no activity.

Diesel exhaust particulates. In collaboration with the DEC Division of Air we are studying the mutagenicity of diesel exhaust particulates. Extracts were prepared and fractionated as previously described (4-6). With the DDA assay we can detect the DNA-damaging activity of as little as 10^{-9} to 10^{-5} μg of diesel extract/ml, in contrast to the 50 to 200 μg needed per plate for routine Ames examination. Thus much smaller quantities of samples need be collected and handled in chemical isolation procedures.

Typical results are shown in Fig. 4 for extracts from an Oldsmobile 350 and a Caterpillar 3304 each furnished dissolved in DMSO by the EPA. Results with the EPA Oldsmobile sample in DMSO were very low in both assays, compared with a solid

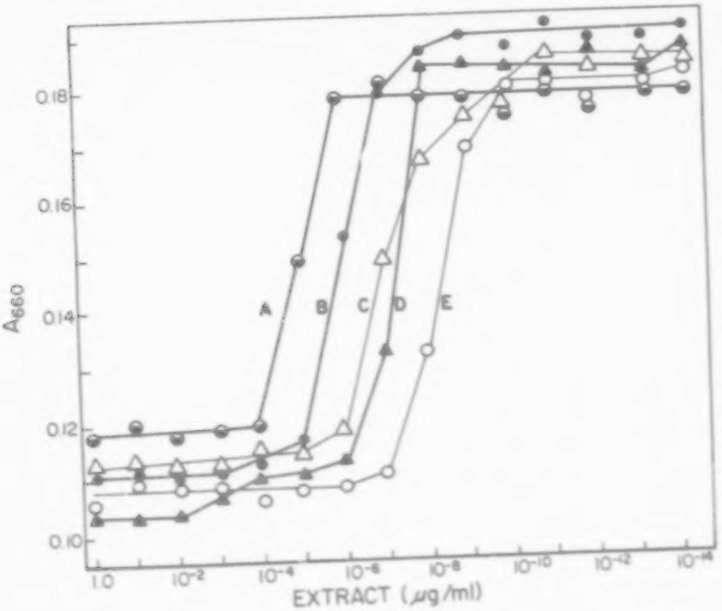

Figure 4. DDA activity of extracts of diesel exhaust particulates from (A) a Caterpiller 3304 (EPA), (B) an Oldsmobile 350 (EPA), (C) a Nissan CN6-33 (DEC), (D) a Volkswagen Rabbit (DEC), and (E) an Oldsmobile 350 (DEC). See Table 2 for Ames test results.

sample subsequently received from the EPA and weighed out and dissolved in DMSO by us. Extracts from exhaust particulates of Nissan CN6-33, Volkswagen Rabbit and Oldsmobile 350 diesel motors showed considerably greater activity. Results with these extracts in the Ames test (Table 2) corresponded roughly with the DDA assay data, suggesting a reasonable agreement between mutagenicity and the DNA damage measured by this assay.

TABLE 2. MUTAGENICITY IN THE AMES TEST OF 100 μg OF THE DIESEL PARTICULATE EXTRACTS SHOWN IN FIG. 4*

	Strain	
Source	TA98	TA100
Caterpiller 3304 (EPA)	0	32
Nissan CN6-33 (DEC)	390	1321
Oldsmobile 350 (EPA)	94	180
Oldsmobile 350 (DEC)	827	1488
Volkswagen Rabbit (DEC)	150	490
Basic fraction	16	0
Acidic fraction	627	228
Neutral fraction	119	166

*Colonies per plate. S9 not used. See Choudhury and Doudney (4) for details of the fractionation and the Ames assay.

Choudhury and Doudney (4) fractionated the Rabbit diesel extracts. In the DDA assay the acidic and neutral fractions were several orders of magnitude more inhibitory than the basic fraction (Fig. 5). The Ames test showed little mutagenic activity with the basic fraction but considerable mutagenicity with the acidic and neutral fraction (Table 2), again supporting a correspondence between mutagenicity and DNA damage.

DISCUSSION

By measuring the effect of DNA-damaging chemicals on the increase in turbidity of E. coli recA uvrA before inducible DNA repair functions have time to develop, the DDA assay is extremely sensitive in detecting those chemicals.

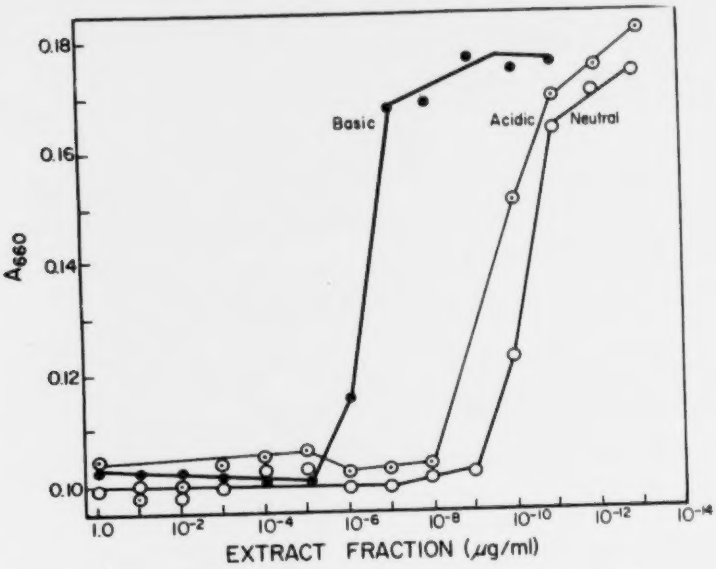

Figure 5. DDA activity of acidic basic and neutral fractions of the Volkswagen Rabbit diesel particulate extract. See Table 2 for Ames test results.

End-point measurements, such as survival, would not achieve a similarly high sensitivity. Such damage presumably is not lethal, as a recovery mechanism exists (3).

The results presented here are preliminary and were obtained primarily to demonstrate the utility of the assay. The biological significance of any given degree of contamination remains unknown, and the specific responses of the DDA assay to representative chemicals likely to contaminate the environment must be studied in detail.

Detection of DNA-damaging chemicals by this system should not be considered to indicate with certainty the presence of mutagenic and carcinogenic agents. Excessively contaminated material should be studied further by concentration, fractionation, and isolation of groups of chemical components, using this test to follow activity; and the isolated DNA-damaging materials should be tested by more traditional methods, such as the Ames test, to confirm mutagenicity.

Nevertheless, the extreme sensitivity of this assay allows a number of approaches toward examination of the environment for contamination not possible heretofore. For example:

Air Particulate Sampling. The DDA assay should greatly reduce the quantity of material needed, so that more frequent monitoring of air for DNA-damaging material is possible.

Mutagenic Vapors. This assay should be sensitive enough for examination of vapors in air. The low levels of potential mutagens found even in severely contaminated air make collection and testing with less sensitive systems impractical.

Surface Contamination. The sensitivity of the DDA assay should make detection of contamination of surfaces by the wipe method practical.

Surface Soil Sampling. The sensitivity of the assay should make testing of extracts of small soil samples for DNA-damaging material practical. Contaminants in soil surface layers are a good indicator of air contamination.

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Session III

BIOCHEMICAL AND METABOLIC EFFECTS OF DIESEL EMISSIONS AND DIESEL EMISSION COMPONENTS

Chairman:

Robert M. Danner

Lung Biochemistry of Rats Chronically Exposed to Diesel Particulates.
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LUNG BIOCHEMISTRY OF RATS
CHRONICALLY EXPOSED TO DIESEL PARTICULATES

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ABSTRACT

Male rats were exposed under comparable experimental conditions to diesel emissions at concentrations of 0, 250 and 1500 $\mu g/m^3$ diesel exhaust particulate, for twenty hours a day and 5-1/2 days per week. After 12, 24 and 36 weeks of exposure, the rats were sacrificed and the lungs analyzed by morphological and biological methods.

Body weight was not changed by the exposure to diesel emissions. Lung wet weight, normalized to body weight, was significantly higher ($p < 0.01$) after 12 weeks of exposure to 1500 $\mu g/m^3$ diesel exhaust particulates. Cell content in the lung tissue (DNA) was significantly increased at 1500 $\mu g/m^3$ after six months. The rate of collagen synthesis was significantly increased while collagen deposition was not affected. Total lung collagen content increased proportionately with the change in lung weight. Prolyl hydroxylase was increased only after 12 weeks exposure and its activity decreased with the age of the rats. A significant increase in lipids (phospholipids and cholesterol) was found in rats exposed for 36 weeks at 1500 $\mu g/m^3$. The profile of fatty acids was not significantly changed.

The results suggest that due to the exposure of rats to diesel emissions, lipids accumulate within the lung tissue

and the reactivity of fibrogenic cells is enhanced. The increased rate of collagen synthesis is compensated by increased collagen degradation, resulting in no net collagen accumulation in the lung during the investigated time period.

INTRODUCTION

Current views on the reactivity of lung tissue to inhaled particles and vapors changed substantially with the introduction of new scientific techniques to ascertain tissue injury. It has been documented that exposure of lungs to respirable particles of classical inert dusts such as TiO_2 induce cellular reactions comparable with transient inflammatory changes [1]. Intratracheal instillation of saline induced significant changes in lung lipids, non-collagenous proteins, and DNA, which returned to normal within 48 hours [2,3]. Finally, it has been recognized that lung (similar to liver tissue), plays an important role in metabolism of various xenobiotics [4]. Accordingly, we can expect that biochemical changes will be observed in the lungs of rats chronically exposed to high concentrations of diesel particulates. The nature, magnitude, and the dynamics of occurrence of these changes was the objective of this study.

MATERIALS AND METHODS

The experimental protocol dealing with lung biochemistry is outlined in the following Tables 1 and 2. In brief, young adult male rats, eight in each group, were exposed for 12, 24 and 36 weeks, 20 hours daily, 5-1/2 days per week, to 250 or 1500 $\mu g/m^3$ of diesel exhaust particulates. A control group was exposed in an identical environment to ambient air. At time of sacrifice, body weight and lung wet weight were recorded. The content of DNA was determined [5] as a measure of cell content and hydroxyproline was determined [6] as a measure of collagen in the homogenized tissue. We also studied noncollagenous proteins and some lipids [8], namely, phospholipids [9], cholesterol [10], and the fatty acid profile. Among dynamic parameters of tissue injury, we measured the rate of collagen biosynthesis [11], and the activity of prolyl hydroxylase [12,13]. In the experience of our laboratory, the latter reflects the functional state of fibrogenic cells. We also analyzed the activity of lysyl oxidase, an enzyme which stabilizes collagen molecules by covalently cross-linking them [14]. The increased activity of this enzyme quite often coincides with the inflammatory process. The changes in all these parameters of lung chemistry were related either to total lung weight (absolute changes) or expressed per gram of lung tissue (density changes). Finally, in order to obtain a meaningful biological insight into the changing lung chemistry, we plotted the results against various reference bases, such as lung weight, DNA content, or ratios of the above parameters. Anywhere a reference is made to statistical significance in the results, these are related to appropriate control group and refer to values of $p < 0.01$ unless otherwise indicated.

Table 1

Experimental Design

Exposure Dose ↓ / Time →	12	24	36
Control		Total 72 rats	
250 $\mu g/m^3$			
1500 $\mu g/m^3$			

Exposure 20 hours/day, 5-1/2 days/week.
Rats - *Rattus norvegicus* from Charles River
Young adult males

Table 2

Experimental Design

Lung Parameters Studied	Reference
Body weight	
Lung wet weight	
DNA	Burton (1956) [5]
Collagen (Hydroxyproline)	Stegemann (1958) [6]
Noncollagenous Protein	Lowry et al (1951) [7]
Lipids - Phospholipids	Folch (1951) [8] Raheja et al (1973) [9]
Cholesterol	Zak (1965) [10]
Fatty Acid Profile	GLC method
Fluorescent Product	Tappel (1973) [15]
Rate of Collagen Synthesis	Juva and Prockop (1966) [11]
Prolyl Hydroxylase Activity	Hutton et al (1966) [12] Bentley and Weiser (1976) [13]
Lysyl Oxidase Activity	Pinell and Martin (1968) [14]

RESULTS

Body weights of rats in all groups increased significantly during 36 weeks of the experiment; however, no adverse effects of diesel particulate exposure was observed within any one time period (Table 3).

Lung wet weights, absolute or relative (related to 100 g body weight), were significantly higher in rats exposed to 1500 $\mu g/m^3$ diesel particulates at all these sampling periods. No significant changes in lung weight were observed in 250 $\mu g/m^3$ exposed group (Table 3).

We analyzed the possible reasons for a significant increase of wet weights of lungs of rats exposed to 1500 $\mu g/m^3$ D.P. The most logical explanation for such an increase would be the accumulation of tissue water in the interstitium due to an increased vessel wall permeability. Direct determination of tissue water in these groups of rats as compared to control lung showed, however, no edema formation, the water content forming 77.9% in the exposed lungs and 78.4% in the controls.

Table 3

Effect of Chronic Exposure to Two Concentrations of Diesel Exhaust Particulates on Body and Lung Weight of Rats

BODY WEIGHTS (g)

Weeks	Concentration diesel particulate ($\mu g/m^3$) 0	250	1500
12	346.5 ± 15.8	336.4 ± 22.5	343.2 ± 17.9
24	363.1 ± 25.1	374.8 ± 15.0	376.4 ± 19.2
36	397.6 ± 17.7	414.6 ± 33.4	403.8 ± 19.9

LUNG WEIGHTS - RIGHT LOBE (g)

Weeks	0	250	1500
12	0.7118 ± 0.0354	0.6980 ± 0.0408	0.7508 ± 0.0485
24	0.7408 ± 0.0461	0.7477 ± 0.0238	0.8444 ± 0.0453*
36	0.7571 ± 0.0416	0.8039 ± 0.0516	1.012 ± 0.0709*

LUNG WEIGHT - RIGHT LOBE/BODY WEIGHT (g/100g)

Weeks	0	250	1500
12	0.2055 ± 0.0091	0.2078 ± 0.0085	0.2188 ± 0.0076*
24	0.2041 ± 0.0044	0.1998 ± 0.0078	0.2244 ± 0.0065*
36	0.1904 ± 0.0082	0.1965 ± 0.0072	0.2506 ± 0.0098*

Variability given by $\bar{x}$ ± S.D. *$p > .01$.

Cellular content (DNA) of the lung was unchanged at 250 $\mu g/m^3$ exposure compared to controls for the entire observation period of 36 weeks (Fig. 1A). Surprisingly, exposure to 250 $\mu g/m^3$ for three months indicated slightly reduced cell content of the lung; the difference was of a borderline significance. In contrast, the DNA content of the lung was significantly increased in high exposures to diesel particulates after 24 and 36 weeks and indicated an increased number of cells in the lung. High level of deposited particulates obviously leads to a hyperplastic cellular stimulation of the respiratory system. Concurrent cytological studies reported in another paper in this symposium indicate a several-fold increase in the number of cells freely migrating in the alveolar lumen, mainly pulmonary alveolar macrophages, and, later on, also neutrophilic phagocytes at the same exposure level. No difference in cell (DNA) density was found in any group at any time interval studied (Fig. 1B).

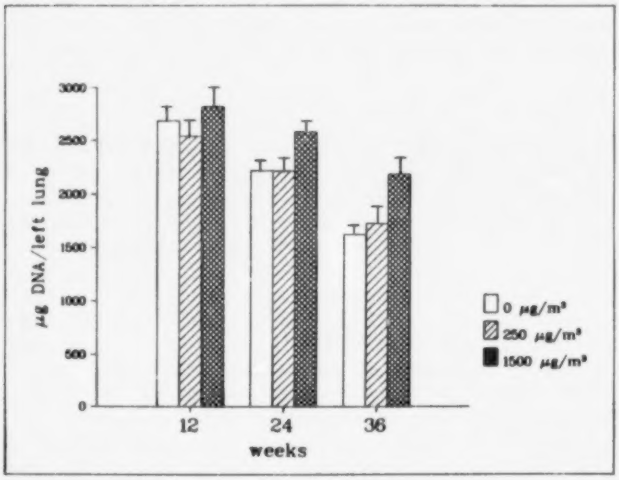

Figure 1A Total DNA contained in the left lung lobe. Total DNA in the left lobe was determined using the method of Burton (1956) [5].

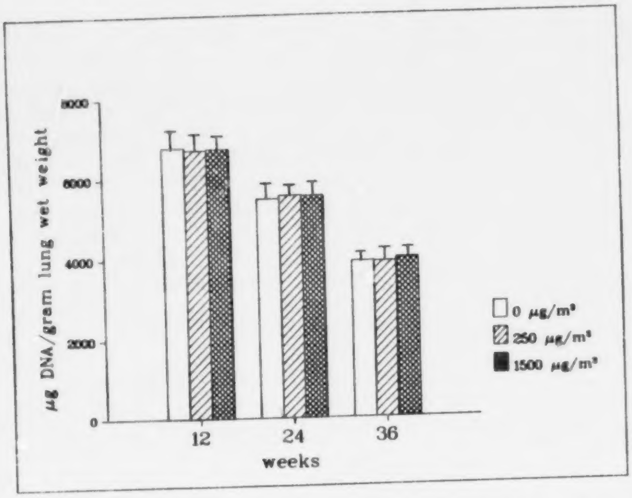

Figure 1B Density of DNA in the left lung lobe. The density of DNA in the left lobe is expressed as μg/g wet weight. Determinations were done on rat lungs exposed to 0, 250, and 1500 μg/m^3 diesel particulates at 12, 24 and 36 weeks.

Rate of collagen synthesis was significantly increased only at the highest exposure (1500 μg/m^3) to diesel particles after 12, 24, and 36 weeks. The magnitude of overall synthetic rate decreases slightly with the duration of the exposure, that is, with the age of the exposed animals (Fig. 2). The increased production of collagen at 1500 μg/m^3 did not result, however, in net collagen deposition in the lung, as both absolute amount (Fig. 3A) and density (Fig. 3B) of this fibrous protein were significantly lower in almost all experimental groups when compared to appropriate controls. On the other hand, no significant changes in the rate of collagen synthesis were detected in the low level (250 μg/m^3) exposure group (Fig. 2), in spite of the accumulation of milligram amounts of diesel particulates and highly pronounced discoloration of the lung.

The specific activity of prolyl hydroxylase was significantly elevated in both experimental groups only at 12 weeks (Fig. 4).

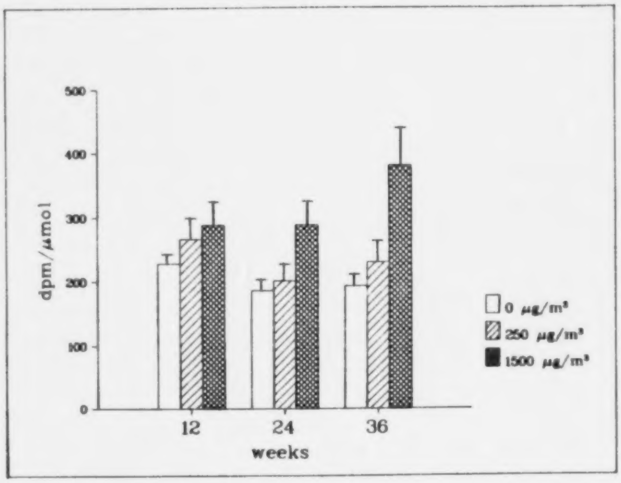

Figure 2 Rate of Collagen Biosynthesis. The specific activity, dpm/μmol, of ^{14}C-hydroxyproline was determined using the method of Juva-Prockop (1966) [11]. Samples were obtained from rat lungs exposed to 0, 250 and 1500 μg/m^3 diesel particulates for 12, 24 and 36 weeks.

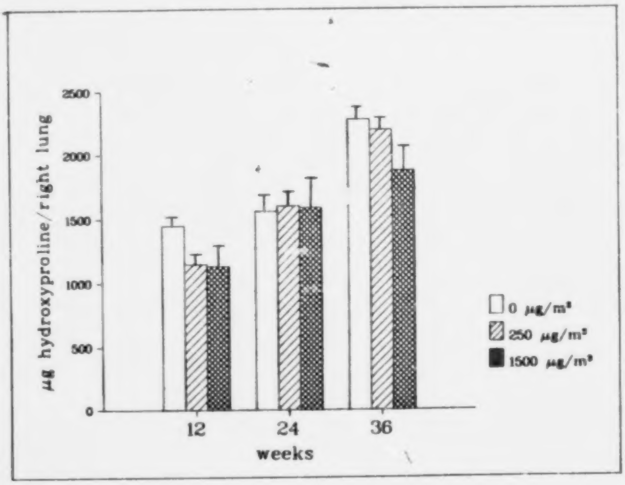

Figure 3A Total Collagen in the right lung lobe. The total collagen contained in the right lobe of rats exposed to 0, 250 and 1500 μg/m^3 diesel particulate for 12, 24 and 36 weeks was determined as hydroxyproline using the method of Stegemann (1958) [6].

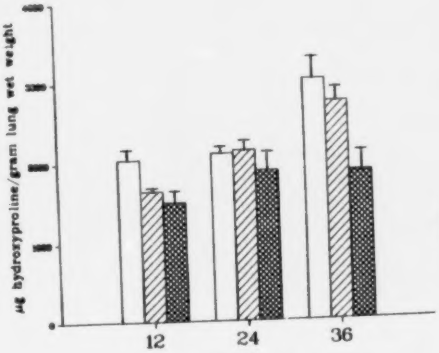

Figure 3B Density of collagen in the right lung lobe. The density of collagen in the right lobe is expressed as hydroxyproline, μg/g wet weight.

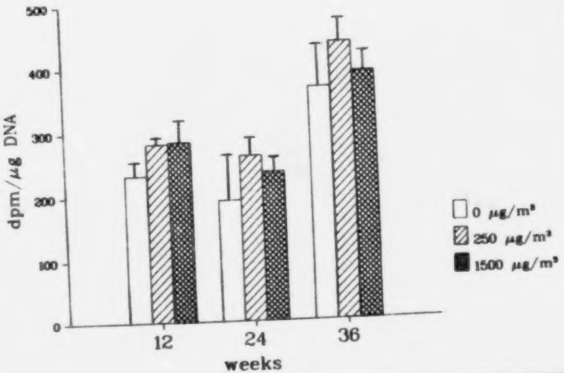

Figure 4 The specific activity of prolyl hydroxylase. The specific activity of prolyl hydroxylase, dpm/μgDNA, was determined using the method of Hutton et al (1966) [12], as modified by Bentley and Weiser (1976) [13].

Phospholipids and cholesterol were both significantly increased after 36 weeks of exposure to 1500 μg/m^3 diesel particulates when related to whole lung. In addition, phospholipids at 24 weeks were also significant at $p > 0.05$ at this particulate concentration. The density of these lipid species followed exactly the same pattern (Table 4). In contrast, no significant differences were observed in the 250 μg/m^3 group in either analysis.

The profile of fatty acids showed significant reduction of saturated as well as polyunsaturated fatty acids in 12 week exposures to 250 as well as to 1500 μg/m^3 particulates. No changes were observed after 24 or 36 weeks of the exposure.

Table 4

Lipid Content in the Lung of
Control and Diesel Particulate Exposed Rats

	PHOSPHOLIPIDS mg/g wet weight left lobe		
	Diesel particulate (μg/m^3)		
Time (weeks)	0	250	1500
12	22.426 ± 3.710	19.792 ± 2.114	22.106 ± 3.924
24	22.441 ± 2.299	21.686 ± 2.622	27.207 ± 3.945*
36	21.718 ± 1.802	21.918 ± 2.588	29.580 ± 2.599**
	CHOLESTEROL mg/g wet weight left lobe		
12	5.469 ± 0.406	5.000 ± 0.582	5.024 ± 0.537
24	7.090 ± 0.563	6.943 ± 0.330	7.566 ± 0.894
36	5.265 ± 0.488	5.582 ± 0.604	6.732 ± 0.631**
	FLUORESCENT PRODUCT relative fluorescence/g wet weight left lobe		
12	13.63 ± 1.98	14.92 ± 1.92	16.30 ± 2.25*

* $p > .05$
** $p > .01$

DISCUSSION

An analysis of the described changes in lung chemistry, although very complex, indicates that the lung can tolerate the exposures to concentrations of diesel particulates as high as 250 $\mu g/m^3$ for more than 36 weeks, without any chemical tissue reaction. This happens in spite of the accumulation of large quantities of particulate matter in the respiratory system. Particulate burden of the lungs was estimated at approximately 3-4 mg of particulates for this concentration at 36 week exposure.

Only with excessive exposures (lung particulate burden in excess of 7-8 mg) do we observe a biochemical reaction. It is manifested by an increased cellularity of the lung (produced in part by the mobilization of cellular macrophages, in particular alveolar macrophages and neutrophils in the alveolar lumen) and increased rate of collagen synthesis. There are also significant increases in noncollagenous proteins (data not shown), phospholipids and cholesterol. Interestingly, these reactions are accompanied by changes in the specific activity of prolyl hydroxylase which becomes significant after three months of exposure at the dose of 250 $\mu g/m^3$ and probably reflects transient stimulation of fibroblasts, probably due to a specific fibroblast stimulating factor released by pulmonary macrophages.

The rate of collagen synthesis was increased only after exposures to high concentrations of diesel particulates and was not, at all, elevated at the low level exposures. No specific inflammatory changes other than the increase in noncollagenous proteins and lipids, which are normally observed as a transient impact after inhalation of inert dusts or intratracheal instillation of saline were found at any exposure level or exposure time. The reasons why we have difficulty in characterizing the changes in lung biochemistry as inflammatory changes are based on the following findings:

a. no increased water content in the lung
b. no cell infiltration with increase of cell density (DNA/weight)
c. only early transient increase in the activity of prolyl hydroxylase
d. no increased activity of lysyl oxidase.

Several chemical parameters of tissue injury showed significant deviation from controls only at the early time exposure and returned to normal values after prolonged exposure. These were prolyl hydroxylase activity, profile of fatty acids and accumulation of fluorescent products [15]. Although it may be difficult to interpret the meaning of such a transient change, we speculate that the lung reacts to the early encounter with diesel exhaust particulates by compensatory cell activation and proliferation of the whole lung tissue. The cytodynamics of this type of lung injury is reminiscent of the reparative changes in the lung after oxygen exposure as reported by Bowden and Adamson [16]. They also observed depression of cellular activity with longer exposure times.

The morphology of the lungs at 1500 $\mu g/m^3$ exposure will be reported later in this session and showed local inflammatory lesions in the peribronchial tissue. This reaction may explain the increased content of granulocytes as well as alveolar macrophages in the lavaged fluid from this group of rats. The finding of inflammatory foci in the lung interstitial tissue appeared to be restricted and did not result in significant changes in lung chemistry, indicating a diffuse inflammatory reaction. Furthermore, several presentations at this Symposium documented that none of the pulmonary function tests of animals exposed to diesel exhaust particulates were changed, as would be expected in tissue modified by the inflammatory process. Thus, the biochemical changes reported in this study are further corroborated by morphological picture and evaluation of the lung functions. The complex analysis seems to indicate that the proliferation of the lung cells is only a compensatory reaction to the presence of diesel exhaust particulates in the alveoli.

It may, therefore, be concluded that only under conditions of massive deposits of diesel particulates in the respiratory system can we observe a slow dose-dependent proliferative reaction. Lungs exposed to the low dose of diesel particulates showed the first trend for change only after a prolonged period of 36 weeks of the exposure.

The increases in lung wet weight, non-collagenous proteins and lipids, and the accumulation of fluorescent product may reflect a pattern usually found in non-specific inflammatory processes. A fibroproliferative process, on the other hand, is documented by increased specific activity of prolyl hydroxylase and the increased rate of collagen synthesis. Our results show that in spite of increased collagen synthesis, there is no abnormal collagen deposition in the lung. The observed change in the synthetic rate indicates, therefore, increased collagen turnover. Presently, we are testing the hypothesis that there is an overall increase of collagen turnover by specific animal experiments and by experiments with fibroblasts grown in tissue culture incubated with diesel particles.

In order to illustrate the magnitude and dynamics of the reported changes in lungs exposed to diesel particles, we would like to show briefly other results presenting similar biochemical changes in the lungs after various doses of

Table 5

EFFECT OF VARIOUS DOSE OF SILICA ON LUNG CHEMISTRY SIX DAYS AFTER ADMINISTRATION*

	Dose of Quartz (mg/rat)				
Parameter Studied	0	10	30	50	75
Lung Weight (g)	1.28 ± 0.03	2.05 ± 0.08	3.25 ± 0.09	4.40 ± 0.32	5.16 ± 0.44
DNA†	7.95 ± 0.42	12.60 ± 1.84	14.86 ± 0.95	18.91 ± 0.82	20.55 ± 1.68
Hyp✓	2.60 ± 0.26	3.31 ± 0.62	4.34 ± 0.69	6.80 ± 0.65	7.88 ± 1.06
Noncollagenous proteins †	88.60 ± 9.81	189.1 ± 29.2	239.8 ± 8.5	307.6 ± 37.2	357.4 ± 45.0
Lipids†	58.51 ± 7.5	153.6 ± 17.2	201.0 ± 12.6	195.5 ± 11.2	190.3 ± 10.2

* There were six to nine male rats, 250 g body weight, in each group.

Variability is given by $\bar{x}$ ± SE.

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† The data are presented in mg/lung.

✓ Hydroxyproline (Hyp) x 7.46 equals the amount of collagen in the whole lung.

quartz (Table 5). Note that within 6 days after a single intratracheal administration of the smallest dose (10 mg) of the standardized fibrogenic silica dust DQ-12, much more pronounced changes occurred than after exposure to 1500 $\mu g/m^3$ diesel particles for 36 weeks. Since we cannot directly compare the effects of a single intratracheal injection with chronic exposure, this comparison is not meant to minimize the potential risk of the inhalation of diesel particulates on the lung, but to show the observed findings in perspective to the well-known fibrogenic effect of silica particles.

It seems that our findings produced more new questions than answers to existing problems. We certainly would like to know the possible contribution of benzo[a]pyrene metabolism by the lung mixed function oxidases to the susceptibility of the tissue exposed to particulates. Another important question is the role of the possible involvement of lung macrophages in the development of the fibrotic lesion [17-20]. Evidence was presented at this symposium that lung macrophages are activated by diesel particulates. Other evidence suggests that activated macrophages produce substances promoting the activity of fibrogenic cells. Obviously, we can expect that many of these mechanisms respond to the deposited mass of particulates. More research is needed before the observed subtle changes can be described in detail, evaluated, and assessed in the light of the expected exposure levels to diesel exhaust on our roads.

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General Discussion

G. STONER: Many of the reactions that you have talked about are classic reactions which occur when there is injury to the peripheral lung. However, at least one that you didn't mention was changes in type II cells. Do you see a type II cell hyperplasia such as you commonly see when you injure the peripheral lung?

M. CHVAPIL: I believe that there is an inflammation located mostly in the bronchial tree. There is an increase in polymorphonuclear leucocytes not only in the lavage system, but also in the bronchial tissue. Therefore, there is a typical reaction only after the intratracheal administraton of 15,000 micrograms of silicon particle. Some changes in the type II cells have been related, but I was not involved in this type of research.

G. STONER: Would an increase in some of the phospholipid profiles be suggestive of that change?

M. CHVAPIL: I believe the phosphate deposited in the lungs comes primarily from the liver. We recently have shown that the liver recognizes injury to the lung within a few hours and the enormous lipid centers in the liver are active in the producton and trannsport of these lipids. We just did a study, and the liver seems to be the major producer of lipids not relating to the surface active lipids.

G. STONER: Do you see any changes in the bronchiolar epithelium and hyperplasia?

M. CHVAPIL: Again the same answer. There will be the morphological study of injuries to explain this. For example, rats exposed to low oxygen tension will definitely have an increase in the fibrinogenic subpopulation in the lung. That has been shown. There is a cellular hyperplasia. If you have an increased population of fibrinogenic cells you would also expect that they will produce more collagen. In fact, local tissue hypoxia has been recognized as a fibrinogenic stimulus. Therefore, I believe hypoxia should promote and potentiate the fibrinogenic reaction to any stimulus.

BURNS: It looks like your changes could be due just to a simple hypoxia. It is unclear where it is coming from, but could it happen that way?

M. CHVAPIL: I believe your suppositions of hypoxia wouldn't apply in this case because we couldn't see anything that would indicate a hypoxia reaction. You don't want to overload your lungs with diesel particles; 75 milligrams of silicate dumped into the lung is an absolutely huge amount which cannot be compared with anything that you are doing with inhalation studies. What I am trying to say is that the changes I am finding with silicate in six days are enormous as compared with those we are finding in nine months. In regard to your first comment, after the highest dose there is a bronchial inflammation. Otherwise, I haven't seen it myself.

DNA-BINDING STUDIES
WITH DIESEL EXHAUST PARTICLE EXTRACT

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ABSTRACT

This study examines whether chemical components from diesel exhaust particulates react with DNA to form covalently bound adducts. Experiments in this report describe the *in vitro* reaction of purified DNA with a dichloromethane extract of diesel exhaust particulates in the absence or presence of enzyme activation by rat liver microsomes. The reactivity of the particle extract was compared to that of benzo[a]pyrene metabolites using low temperature fluorescence techniques which detect small quantities of polycyclic aromatic compounds bound to DNA. Incubation of DNA with the particle extract in the presence of microsomal enzymes produced no detectable fluorescent adducts in contrast to model experiments using benzo[a]pyrene. However, addition of the particle extract to incubation mixtures containing benzo[a]pyrene markedly decreased formation of benzo[a]pyrene-DNA adducts because the particle extract inhibits microsomal enzymes which activate benzo[a]pyrene and other polycyclic aromatic hydrocarbons. In the absence of microsomal enzymes, fluorescent material was detected in DNA exposed to high concentrations of the particle extract, but probably not as a result of covalent binding because the mutagenic activity of the particle extract remained unchanged during prolonged incubation with DNA. This stability is in contrast to the rapid decrease in mutagenic activity of benzo[a]pyrene-4,5-oxide during incubation with DNA. Thus, direct mutation of bacteria by the particle extract may require activation by bacterial enzymes as is known to occur with nitroaromatic compounds.

INTRODUCTION

Most chemical mutagens and carcinogens induce genetic damage by forming covalent adducts with DNA [1-3]. As a result of such modifications to the structure of DNA, errors can be introduced in a cell's genetic information during DNA replication and cell division. In bacteria or cultured animal cells, these errors may be recognized as genetic mutations. Similar genetic errors are believed to be an initiating event in a series of changes which *in vivo* transform normal cells into tumors [4].

The chemical components extracted from diesel particulates by organic solvents are mutagenic in bacteria [5-7]. These extracts contain benzo[a]pyrene (B[a]P) and other polycyclic aromatic hydrocarbons (PAH) but simple unsubstituted PAH are not the major mutagenic components because the particle extract is mutagenic to bacteria even in the absence of mammalian microsomal enzymes. However, certain substituted PAH such as epoxides and nitro-derivatives are directly mutagenic in bacteria [8-12]. Since the identity of extracted mutagenic components and their biological mechanism of action are unknown, it is difficult to assess what genetic effects would be induced in human or other mammalian tissues.

The DNA binding studies described in this report were all done *in vitro*, by incubating purified DNA with diesel particle extract or reactive metabolites of B[a]P. Since the particle extract contains both unsubstituted PAH and components directly mutagenic to bacteria, the reaction between the extract and DNA was studied in both the presence and absence of the microsomal enzyme system from rat liver. The methods used to detect covalent binding of mutagens to DNA include the low temperature fluorescence techniques first described by Ivanovic and coworkers [13]. Low temperatures prevent fluorescence quenching and allows the fluorescence to be detected in conventional spectrofluorometers. Although restricted to fluorescent adducts, primarily those having condensed aromatic ring structures, the characteristics of excitation and emission spectra provide valuable structural information.

METHODS

Collection and Extraction of Diesel Particulates Detailed procedures for collecting and extracting the diesel particulates have been reported by Chan and Lee [14]. Diesel exhaust particulates were collected by electrostatic precipitation from the undiluted exhaust (at 100°C) of a 1978 5.7 L GM diesel engine built by Oldsmobile (constant speed = 1350 r/min, load = 96 N·m). The extract was prepared by Soxhlet extraction with dichloromethane. Solvent was removed under a stream of nitrogen gas and the extracted material redissolved in dimethylsulfoxide (10 or 100 mg/mL) and stored at -80°C.

Animal Treatment and Microsome Isolation Liver microsomal membranes were prepared from rats (male, Sprague-Dawley, 200-250 g) intraperitoneally injected 48 hours prior to sacrifice with 3-methylcholanthrene (20 mg/kg) dissolved in corn oil. The microsomal fractions were isolated from liver homogenates by differential centrifugation, suspended in a solution containing 50% glycerol and 25 mM Tris-HCl (pH 7.4) and stored at -80°C.

Microsomal Enzyme Activity Assays Metabolism of B[a]P to alkali-extractable metabolites was assayed by the fluorescence method of Nebert and Gelboin [15] in reaction mixtures containing 50 mM Tris-HCl (pH 7.4), 3 mM $MgCl_2$, 0.7 mM NADP, 10 mM glucose-6-phosphate, G-6-P-dehydrogenase (1 unit/mL) and 0.025 to 0.050 mg/mL of microsomal protein. Product formation is expressed as fluorescence equivalents of 3-hydroxy-B[a]P used as an internal standard. Reaction rates were determined from the linear increase in product concentration measured by sequential removal of aliquots from reactions incubated for 15 minutes at 37°C under air in a Dubnoff metabolic shaking incubator.

DNA-Binding Reactions Reaction mixtures used in these DNA-binding studies contained deproteinized salmon sperm DNA (1.0 mg/mL), 100 mM phosphate buffer (pH 7.5), 2.5 mM $MgCl_2$, and 0.1 mM EDTA in a total reaction volume of 4 or 5 mL. Incubations including enzymatic activation also contained rat liver microsomes (1.0 mg/mL), 0.7 mM NADP, 10 mM glucose-6-phosphate, and G-6-P-dehydrogenase (1 unit/mL). The mixtures were incubated for 1 hour at 37°C under air in a covered Dubnoff metabolic shaker. Reactions were stopped by placing the reaction vessels on ice and microsomal membranes were removed by centrifugation at 105 000 *g* for 60 minutes.

Isolation and Purification of DNA All operations in the isolation of DNA were done under subdued light at 0-4°C. DNA was recovered from the incubation mixtures by ethanol precipitation, redissolved in 1/10 SSC (15 mM NaCl, 1.5 mM Na-isocitrate) and extracted three times with ethylacetate. After addition of sodium-4-aminosalicylate (4%, w/v) and NaCl (1%, w/v), the solution was extracted twice with equal volumes of phenol-cresol reagent [16] (100 g phenol, 11 mL water, 14 mL m-cresol and 0.1 g 9-hydroxyguinoline). Remaining traces of the phenol reagent were removed by extraction with diethylether. The DNA was precipitated by ethanol, wound on a glass rod and washed successively in ethanol, hexane and again ethanol. Further purification was achieved by isopycnic centrifugation in cesium chloride gradients containing 17 mM

TrisHCl (pH 7.4) and 9 mM EDTA. Peak fractions were combined and chromatographed on a short column (1.2 by 10 cm) of sephadex G-75 and preequilibrated with 60% EGW (60 mL ethylene glycol, 40 mL 1/10 SSC). The DNA therefore eluted from the column in the 60% EGW solvent. The concentration of DNA, determined by its absorbance at 260 nm, was usually between 0.3 and 0.5 mg/mL.

Low-Temperature Fluorescence Measurements The equipment used to measure the fluorescence of DNA samples in 60% EGW at subzero temperatures is illustrated in Figure 1. The fluorescence instrument is a SLM Model 8000D photon counting spectrofluorometer equipped with double grating emission and single grating excitation monochromators. The sample holder was covered with a foam insulating tape and cooled by a chilled stream of nitrogen gas regulated at temperatures from ambient to -190°C. The nitrogen gas exhausts around a cylindrical quartz sample cell (4 mm i.d., 7 mm o.d.) preventing frost formation.

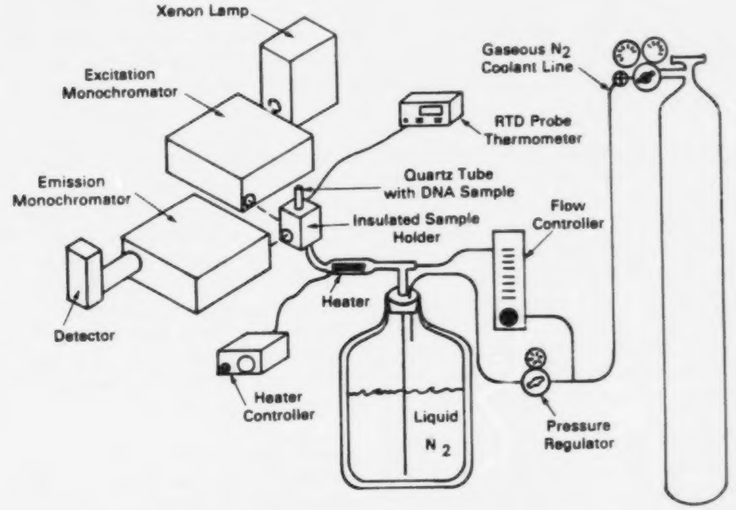

Figure 1 Cryogenic equipment for low-temperature fluorescence measurements.

Spectral Corrections All fluorescence spectra were normalized to exciting light intensity with the response of the reference photomultiplier corrected by use of a quantum counter (rhodamine B). Excitation spectra using broad-band emission were recorded with excitation wavelengths transmitted by a Corning 7-54 filter and emitted light transmitted by a long-pass filter with 50% transmission at 370 nm. Digitized spectra, recorded by a HP9815 calculator and transferred to the GM Research Laboratories computer center, were further corrected for background luminescence from the cell, solvent and the inherent fluorescence of DNA. Fluorescence intensities are described in either arbitrary units or quantitative units (PFU) obtained by using pyrene as an internal standard. The PFU is defined as the fluorescence intensity of 1 nM pyrene in 60% EGW measured under the same conditions as the DNA samples with excitation at 338 nm and emission at 372 nm or over a broad-band spectrum as already described.

Chemicals 3-Methylcholanthrene, sodium-4-aminosalicylate, m-cresol, 8-hydroxyquinoline, bovine serum albumin, NADP, glucose-6-phosphate, G-6-P-dehydrogenase (Type XI), cesium chloride (optical grade) and salmon sperm DNA (Type III) were obtained from Sigma Chemical Company. Benzo[a]pyrene (Gold Label) and 2-nitrofluorene were purchased from Aldrich Chemical Company. Ethylene glycol (Baker Analyzed) and phenol were obtained from J. T. Baker Chemical Company. A sample of 3-hydroxybenzo[a]pyrene was obtained from Dr. C. A. King of the Michigan Cancer Foundation and benzo[a]pyrene-4,5-oxide was obtained from Dr. J. J. McCormick at Michigan State University. Phenol and m-cresol were redistilled and benzo[a]pyrene was recrystallized from toluene-methanol (1:1). DNA was deproteinized by extraction with phenol-cresol reagent. All other chemicals were reagent grade.

RESULTS AND DISCUSSION

Fluorescence Enhancement at Low Temperatures The low temperature fluorescence spectra described by Ivanovic et al [13] were measured at the temperature of liquid nitrogen, -196°C. In this study, fluorescence enhancement was investigated at several temperatures between +20 and -190°C. Figure 2 shows the emission spectra of two samples - one containing pyrene and the other DNA exposed to B[a]P and liver microsomal enzymes. At room temperature, pyrene exhibits a well defined emission spectra but no distinct spectral peaks are discernable in the fluorescence from the DNA-B[a]P sample. As the samples are cooled to -100°C, the 60% EGW solvent is transformed from a liquid to a rigid transparent glass. At this temperature, a distinct fluorescence spectra is observed in the DNA-B[a]P sample with peak emissions at 379 and 399 nm. Below -100°C the fluorescence intensity of the DNA-B[a]P sample increases only slightly in contrast to the fluorescence enhancement with the sample containing pyrene. Since the measurement of fluorescence spectra at -100°C presents less operational problems than lower temperatures, the spectra of DNA samples described in the remainder of this report were measured at -100°C.

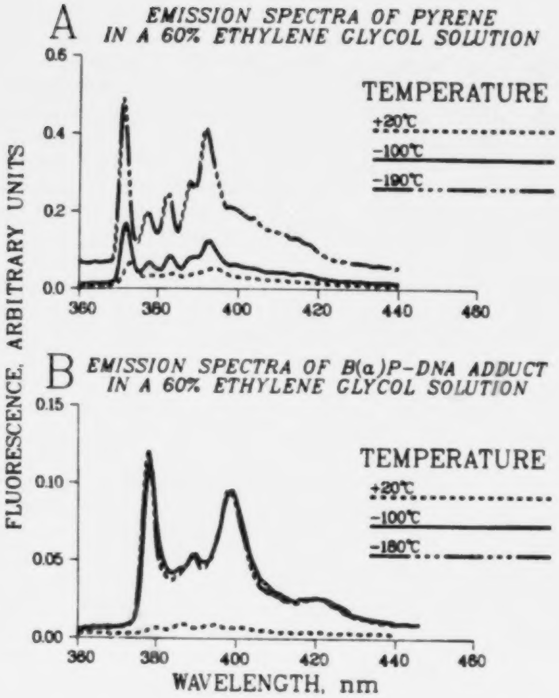

Figure 2 Fluorescence emission spectra at ambient and subzero temperatures. A - spectra of a 10 nM pyrene sample excited at 338 nm. B - spectra of DNA incubated with 10 μM benzo[a]pyrene and microsomal enzymes as described under Methods. The excitation wavelength used for the B[a]P-DNA sample was 348 nm.

Fluorescence Spectra Using Broadband Emission The fluorescence emission spectra shown in Figure 2 were readily measured using excitation maxima previously described by other investigators [13]. To detect other fluorescent adducts in DNA exposed to either B[a]P or diesel particle extract, excitation spectra were recorded while measuring fluorescence emission over a broad band of the visible wavelength range. The fluorescence excitation spectra of the DNA-B[a]P reaction product measured by broad-band emission is shown in Figure 3. Part A shows the uncorrected spectra of three samples; the reaction product; a control sample of DNA; and the solvent and sample cell. The corrected spectra in Figure 3B retains only the fluorescence of the bound B[a]P metabolite(s) expressed in PFU per 10 absorbance units of DNA (~0.5 mg/mL) as described under Methods. The excitation and emission maxima of the DNA-adduct isolated from this reaction mixture are the same as those described for the products formed from the 7,8-dihydrodiol-9,10-epoxide of B[a]P [13,17].

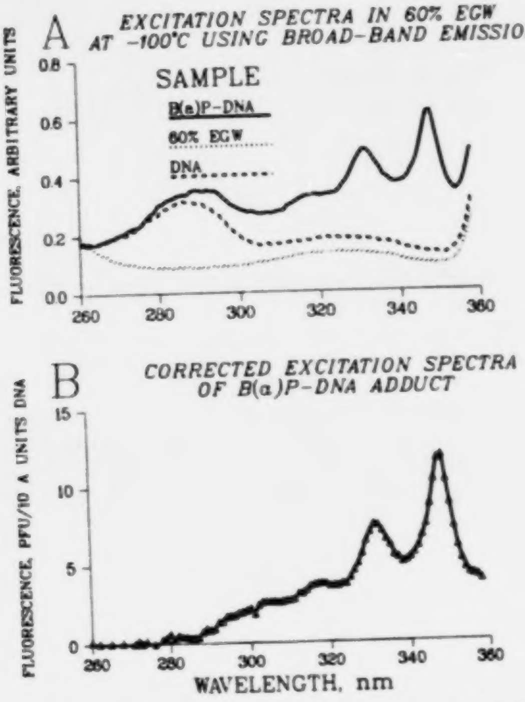

Figure 3 Fluorescence excitation spectra of B[a]P-DNA. A - uncorrected spectra in aribtrary fluorescence units. B - spectrum corrected for luminescence of the solvent cell and DNA expressed in pyrene fluorescence units.

Reaction of DNA with Diesel Particle Extract in Presence of Liver Microsomes DNA was exposed to diesel particle extract at concentrations of 0.1, 1 and 5 mg/mL in the presence of liver microsomes under the same conditions used to prepare the DNA adducts of benzo[a]pyrene. The fluorescence excitation spectra of DNA recovered from these incubation mixtures are shown in Figure 4. There are no discernable fluorescent components in these spectra which are shown in quantitative comparison to the fluorescence spectrum of the DNA adduct of B[a]P. The amount of B[a]P in the particle extract used in these studies has not been measured, but Williams and Chock [18] have reported exhaust particulates from a similar GM 5.7 L engine contains about 10 μg/g of extractable B[a]P. Thus

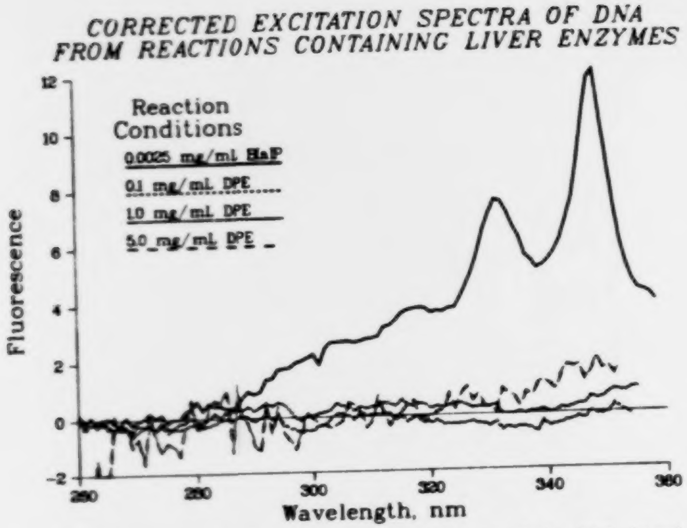

Figure 4 Comparison of fluorescence detected in DNA incubated with diesel particle extract (DPE) or B[a]P in presence of rat liver microsomal enzymes. Other reaction conditions are described under Methods and fluorescence spectra were recorded as in Figure 3B.

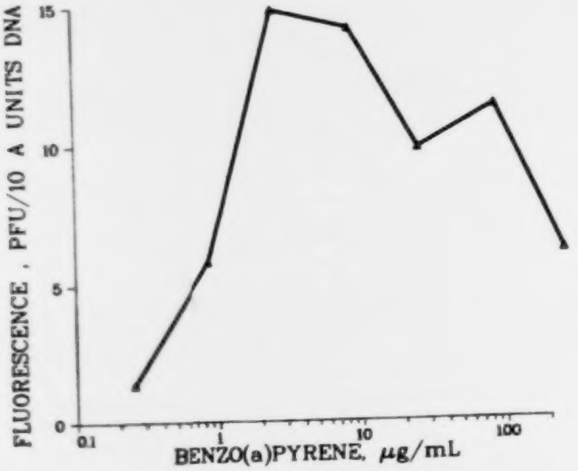

Figure 5 The quantity of fluorescent B[a]P-DNA adduct formed as a function of the concentration of benzo[a]pyrene. Fluorescence emission spectra were recorded as described in Figure 2B at a temperature of -100°C. The values plotted are from the intensities at the 379 nm emission maximum.

DNA binding reactions containing 5 mg/mL of the particle extract should contain nearly 0.5 μg/mL of B[a]P plus other PAH compounds. As shown in Figure 5, the fluorescence of covalently bound B[a]P was detected in DNA exposed to as little as 0.25 μg/mL. Thus the particle extract either contains less B[a]P than estimated, or interferes with the DNA-binding activity of B[a]P.

Inhibition of DNA-Binding Activity by Diesel Particle Extract In Figure 5, the maximal amount of B[a]P-DNA binding activity occurs at concentrations of 2.5 to 10 μg/mL and at higher concentration there is a decrease in binding. This decrease is probably due to the competition between reactions 1 and 2 as shown below. Both reactions are

$$\text{B[a]P} \longrightarrow \text{Primary products including B[a]P-7,8-diol} \qquad (1)$$

$$\text{B[a]P-7,8-diol} \longrightarrow \text{B[a]P-diol-epoxide} \qquad (2)$$

catalyzed by the P450-monoxygenase in liver microsomes. Thus high concentrations of the initial substrate, B[a]P, should inhibit the formation of the diol-epoxide product which subsequently binds to DNA. Other PAH compounds may also inhibit DNA binding activity. Similar inhibition by multicomponent mixtures has been observed in mutagenicity studies with shale oil fractions [19] and model studies have shown one PAH compound may either inhibit or augment the mutagenic action of another [20,21]. Therefore, the effect of the diesel particle extract on formation of DNA adducts of B[a]P was examined.

Figure 6 shows the amount of fluorescent B[a]P adduct detected in DNA from incubation mixtures which include particle extract in addition to B[a]P and liver microsomes. At a concentration of 0.1 mg/mL DPE, there is little change in this DNA-binding activity, but at 1.0 mg/mL, the amount of fluorescent adduct formed is decreased by more than 90%. This decrease in DNA binding activity can be attributed to the inhibition of B[a]P metabolism. Table 1 describes the B[a]P hydroxylase assays which show the inhibition by the particle extract. Since the DNA-binding reactions contained 10 μM B[a]P, the effect of the extract on B[a]P metabolism was measured with this substrate concentration. These assay mixtures also contained 1 mg/mL bovine serum albumin, BSA, as a substitute for the 1 mg/mL of microsomal protein in the DNA-binding reactions. Under these conditions, the inhibition of B[a]P hydroxylase activity is similar to the inhibition of DNA-binding activity. Therefore, the presence in diesel particulates of B[a]P, or other PAH compounds requiring metabolic activation, would probably not be detected in this DNA-binding assay.

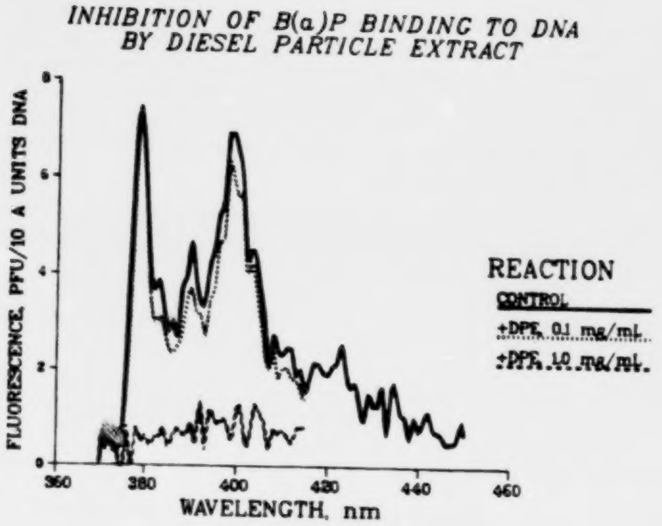

Figure 6 Inhibition of B[a]P-DNA adduct formation in reaction mixtures containing the diesel particulate extract. Each reaction mixture contained 10 μM benzo[a]pyrene and microsomal enzymes. Fluorescence emission spectra were recorded as described in Figure 2B.

Table 1

Inhibition of B[a]P Hydroxylase by the Particle Extract

Reaction Conditions	Benzo[a]pyrene Hydroxylase
	nmol/min/mg
80 μM B[a]P	3.48
10 μM B[a]P*	2.20
+ 0.1 mg/mL DPE†	0.88
+ 0.4 mg/mL DPE	0.13
+ 1.0 mg/mL DPE	0.05

*Reaction mixtures also contain 1.0 mg/mL BSA.
†Diesel particle extract

Direct Reactions of Diesel Particle Extract with DNA in Absence of Liver Microsomes Purified DNA was exposed to the particle extract as previously described except the rat liver microsomal enzymes were omitted from the incubation mixtures. The DNA was isolated from these reaction mixtures and examined for fluorescent adducts. Figure 7 shows the corrected excitation spectra of DNA exposed to three concentrations of the particle extracts. A fluorescent component was observed in DNA from the reaction containing the highest concentration of extract. As shown in Figure 4, this fluorescent component was not observed when DNA had been similarly exposed in the presence of microsomal membranes, and may be a non-covalent association of material in the extract which was not separated by the isolation methods employed.

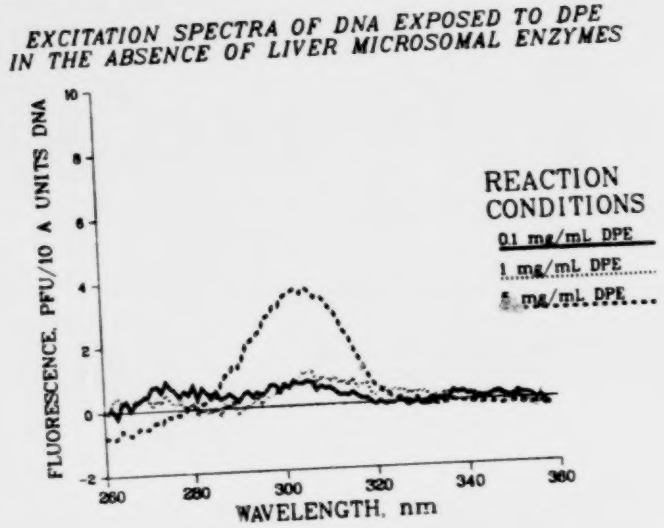

Figure 7 Fluorescence excitation spectra of DNA incubated with diesel particulate extract (DPE) in the absence of an enzyme activation system. Spectra were recorded as described in Figure 3B.

A more definitive characterization of the direct reaction between DNA and the particle extract was obtained by measuring the stability of mutagenic activity during incubation in the presence of DNA. The experimental procedures are illustrated in Figure 8. The mutagenic agent is incubated in either the absence or presence of DNA and at timed intervals, aliquots are removed from the incubation mixtures and assayed for mutagenic activity in the Ames assay as described by Siak et al [22]. If the mutagen reacts covalently with DNA, it will no longer be available to mutate bacteria. These experiments were done using two model compounds, both being direct acting mutagens, but distinctly different in their mechanism of action. One is benzo[a]pyrene-4,5-oxide which reacts directly with DNA to form a covalently bound adduct [17]. The other model compound is 2-nitrofluorene which is not directly reactive towards DNA but is a direct acting mutagen because it is activated by a bacterial nitro-reductase enzyme system [12].

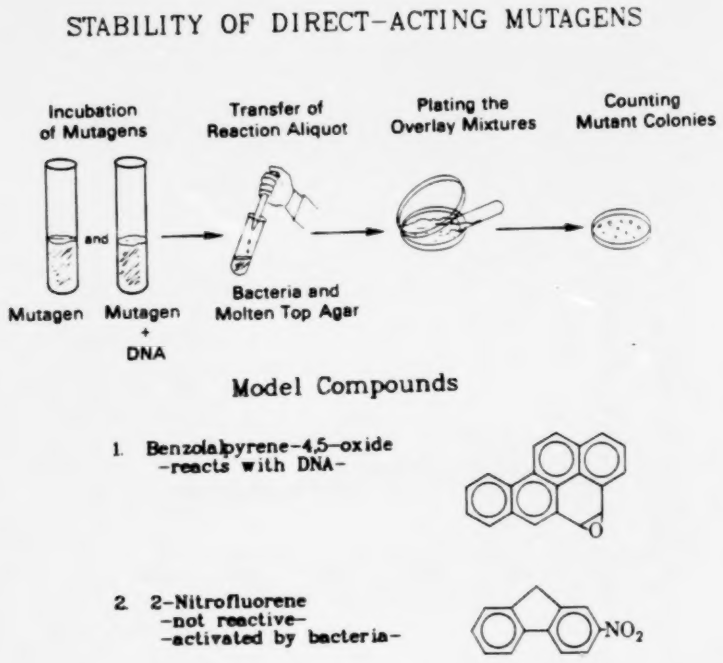

Figure 8 Experimental procedure for examining *in vitro* DNA-binding activity of direct-acting bacterial mutagens.

The results of the experiments with these two model compounds and the diesel particle extract are shown in Figure 9. As expected, benzo[a]pyrene-4,5-oxide rapidly lost mutagenic activity when incubated with DNA in contrast to the slow loss of activity in the control incubation mixture by hydration of the epoxide. The mutagenic activity of 2-nitrofluorene was not decreased at all during the 5 hour incubation with DNA. In a similar experiment with diesel particle extract, the mutagenic activity is not decreased during incubation with DNA. Identical incubation mixtures had been prepared 5 days beforehand which still retained full mutagenic activity.

Therefore, the direct acting bacterial mutagens in the diesel particle extract do not bind covalently to purified DNA.

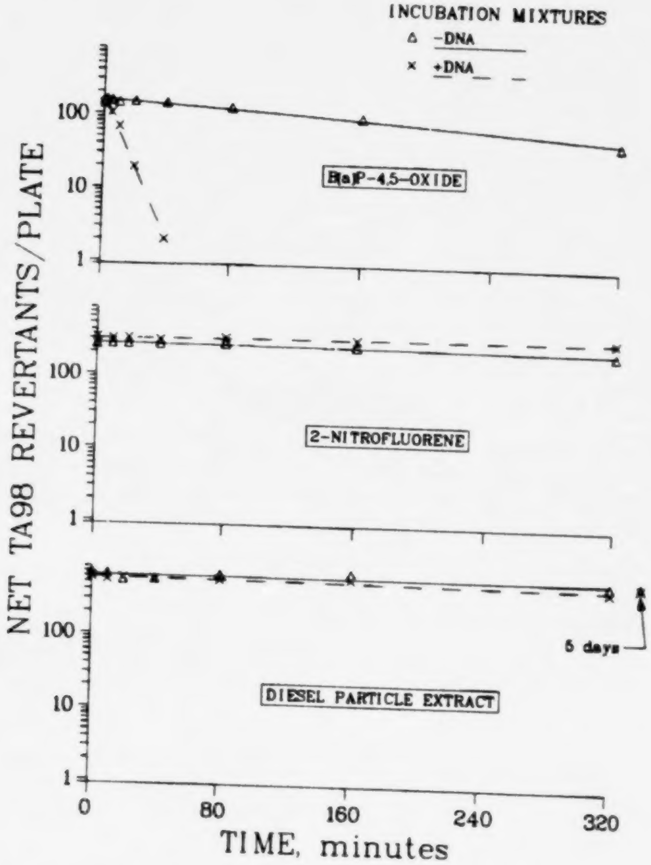

Figure 9 Stability of direct-acting bacterial mutagens incubated in the absence or presence of purified DNA. Mutagenic activity was measured as described in Figure 8 with 0.05 mL aliquots from reaction mixtures containing model compounds (B[a]P-4,5-oxide, 2 μg/mL or 2-nitrofluorene, 20 μg/mL) or diesel particle extract (1 mg/mL) in 1/10 SSC incubated with or without DNA (2 mg/mL) at 37°C.

CONCLUSIONS

This study of reactions between DNA and chemical components extracted from diesel exhaust particulates emphasizes the necessity of further characterizing the specific chemical components and biological mechanisms of action responsible for the activity of diesel particulates in various assays of genetic toxicity. The inhibition by diesel particle extract of microsomal enzymes and binding of B[a]P to DNA illustrates the problems encountered in studying complex mixtures. Such interference by one component of a mixture with the action of another may or may not be of consequence *in vivo*. The direct acting bacterial mutagens in the extract have been shown to not react with purified DNA which implies bacterial enzymes may activate these components as occurs in the mutagenic action of nitroaromatic compounds. Therefore, a detailed understanding of the molecular mechanisms involved in both bacterial and mammalian systems is required to assess any health effects which might be attributed to bacterial mutagens in diesel exhaust particulates.

ACKNOWLEDGEMENT

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General Discussion

D. HOFFMAN: Isn't it possible that your dihydroxide epoxide has reacted with some other "tar constituents" from diesel exhaust? You have not made the control experiment in which you have added the dihydroxide epoxide and that would have shown that there is no binding.

T. PEDERSON: That is true. That is another experiment that could be done, however, it did inhibit AHH activity.

THE EFFECT OF IN VIVO EXPOSURE OF RATS TO DILUTED DIESEL EXHAUST ON MICROSOMAL OXIDATION OF BENZO[α]PYRENE

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ABSTRACT

The effect of exposure of Fischer 344 rats to diesel exhaust at two concentrations, 250 μg/M^3 and 1500 μg/M^3 of diesel particulates on several parameters including the metabolism of benzo[α]pyrene to polar metabolites and to metabolites capable of alkylating denatured DNA has been studied. A years exposure had no effect on the size of the animals or their livers; however after the higher exposure lung weight was increased. After one year, the ability of lung microsomes to oxidize benzo[α]pyrene was impaired at either exposure level. At present it is not certain whether this effect is an artifact due to contamination of the lung microsomes by diesel particles or a genuine biochemical pathology. Liver microsomes seemed to be less able to oxidize the carcinogen at six and twelve weeks after exposure but were no different than control after a years exposure.

INTRODUCTION

Polycyclic aromatic hydrocarbons are environmental pollutants which are formed as products of the incomplete combustion of organic materials. Many of the chemicals are substrates for the mixed function oxidase system located in the enodplasmic reticulum of mammalian cells.

In rats, for example, these chemicals are oxidized to epoxides, phenols and diols by a microsomal electron transport system with spectral characteristics, immunologic properties and catalytic specificities that are different from the usual system responsible for metabolism of xenobiotics (1). Among the products of these oxidations catalyzed by mammalian microsomes are certain epoxide metabolites which have been shown to be mutagenic in *in vitro* assays (2). Benzo[α]pyrene is a carcinogen which has been shown to be a prototypical substrate for this polycyclic aromatic hydrocarbon metabolizing system, and like other substrates for the mixed function oxidases, has the interesting property of being able to induce the enzymes responsible for its own metabolism (3). In fact, there is good evidence that human populations that are exposed to unusually high levels of polycyclic hydrocarbons have an increased level of the microsomal oxidases responsible for benzo[α]pyrene metabolism (4). In our study, we have assayed the activity of the microsomal enzymes involved in benzo[α]pyrene metabolism in both the lungs and livers of Fischer 344 rats which were exposed to two concentrations of diesel exhaust as part of a larger study conducted by the General Motors Corporation. Our objective was to decide whether under the conditions of this study sufficient amounts of the appropriate polycyclic hydrocarbons were introduced via inhalation to produce either a local induction of the microsomal oxidases in lung or a more widespread induction involving the liver as well.

METHODS

Male Fischer 344 rats were exposed at the General Motors Technical Center to clean air or to diesel exhaust diluted with clean air to 250 μg or 1500 μg of diesel particulates per cubic meter. The details of the conditions of the exposure have been described by others (5). After various intervals of time of up to one year, groups of six rats were fasted overnight and then removed from the exposure chamber to be transported about 30 min by auto to our laboratory. The animals were then weighed, decapitated and the liver and lungs were removed, weighed and homogenized in four volumes of 0.25 M sucrose. Mitochondria, nuclei and cell debris were removed by centrifugation at 17400 x g_{max} for 10 min, and microsomes were isolated from the resulting supernatant by centrifugation at 144,000 x g_{max} for 60 min. These microsomal pellets were resuspended in 0.25 M sucrose.

Oxidation of benzo[α]pyrene to polar metabolites was measured by our modification of the method described by Van Cantfort *et al.* (6). Briefly, about .1 mg hepatic or .4 mg pulmonary microsomal protein was incubated for 30 min at 35°C in 0.4 ml of a mixture which contained

1 mM NADP, 6.5 mM sodium isocitrate, 0.05 mM $MnCl_2$, 0.44 international units of pig heart isocitrate dehydrogenase in 50 mM Tris-HCl. Incubations with hepatic microsomes were buffered at pH 7.5 while those with pulmonary microsomes were at pH 8.0. The reaction was initiated by the addition of ^{14}C-benzo[α]pyrene in 10 μl of an acetone solution; the final benzo[α]pyrene concentration was 80 μmolar. Nonenzymatic oxidation of the substrate was estimated for each tissue from each animal by performing a similar incubation except that NADP, isocitrate and isocitrate dehydrogenase were omitted. The reactions were stopped by adding one ml of a mixture of 0.15 N KOH in 85% (v/v) dimethyl sulfoxide. This mixture was twice extracted with three ml of hexane and an aliquot of the aqueous-dimethyl sulfoxide phase was removed and neutralized with acetic acid so that radioactivity could be estimated by liquid scintillation using a commercially available xylene based scintillant. The difference between the amount of radioactivity in the complete incubations and in the incubations without the NADPH generating system was used to calculate the rate of enzymatic oxidation of benzo[α]pyrene. Data are expressed as nmoles of benzo[α]-pyrene oxidized per 30 min incubation per mg microsomal protein and are based on estimates of protein by the Lowry method (7).

A second incubation procedure was used to estimate the ability of the metaholites generated in vitro to alkylate DNA. About 0.4 mg hepatic or 1.0 mg pulmonary microsomal protein was incubated in 2 ml of a mixture of the same composition as was used to assay the oxidation of benzo[α]-pyrene except that 0.5 mg per ml denatured and sheared calf thymus DNA was also present. Incubations were performed in the presence and absence of the NADPH generating system and the reaction was started by adding 50 μl of 3.2 mM ^{14}C-benzo[α]pyrene in acetone. Incubations were allowed to proceed for 60 min at 35°C and then the reaction was stopped by adding sodium dodecysulfate to a final concentration of 1%, and then the mixtures were further incubated for 30 min with 8 mM EDTA and 5 μg per ml ribonuclease A from bovine pancreas. The ribonuclease treated mixture was then extracted with an equal volume (2.5 ml) of water saturated phenol for 30 min at room temperature. The aqueous phase was then mixed with two volumes of 95% ethanol and DNA was precipitated overnight at -18°C. The precipitated DNA was collected by centrifugation at 1000 x g for 10 min and washed twice with 5 ml aliquots of cold 95% ethanol. Finally, the precipitate was dissolved in 1 ml of water; a small protion was removed to estimate the absorbance at 260 nmeters and the remainder was used to estimate the amount of covalently found radioactivity by liquid scintillation. Data are expressed as nmoles of benzo[α]pyrene bound to a mg of DNA per hr per mg of microsomal protein. Protein was determined by the Lowry procedure (7), and calculations of the recovery of DNA were based on the assumption that one mg of DNA absorbs 20 A_{250} over a cm light path.

[7, 10-^{14}C]Benzo[α]pyrene (5-15 Ci/mole) was purchased from Amersham Corporation as a benzene solution. The radiochemical was subsequently dried under a stream of N_2 and redissolved in 3 ml of hexane. The hexane was extracted with 3 ml 0.5 N NaOH in 80% ethanol followed by an extraction with 3 ml 1 N NaOH. Finally, the hexane layer was collected, evaporated under N_2 and the residue was dissolved in an acetone solution of 3.2 mM unlabeled benzo[α]pyrene. The benzo[α]pyrene was stored in the dark at -18°C. Other chemicals were purchased from either the Sigma Chemical Company or the BioRad Corporation.

RESULTS AND DISCUSSION

Fischer 344 rats were exposed to either clean air or dilutions of diesel exhaust containing 250 μg per M^3 or 1500 μg per M^3 of diesel particulates. The conditions of this exposure have been detailed elsewhere (5). At intervals of 6, 12, 24 and 53 weeks animals were removed and body, liver and lung weights were estimated; microsomal fractions were prepared from liver and lungs and were used to estimate the ability of the animals to oxidize benzo[α]pyrene to more polar metabolites and to metabolites capable of covalently reacting with DNA.

In Table 1 the body weights for the three groups are shown, and it can be seen that while the average weight gain over the exposure period was somewhat less in the group exposed to the highest concentration of exhaust, there were not substantial differences between the average body weights at any of the intervals tested. The data in Table 2

TABLE 1

THE EFFECT OF EXPOSURE TO DIESEL EXHAUST ON RAT BODY WEIGHT (GRAMS)*

	Concentration of Diesel Particles		
Duration	Control	250μg/m^3	1500μg/m^3
6 weeks	251 ± 3	249 ± 6	261 ± 4
12 weeks	284 ± 6	297 ± 6	299 ± 3
24 weeks	335 ± 10	364 ± 9	335 ± 6
53 weeks	391 ± 14	392 ± 10	366 ± 17

*Each value is the mean ± S.E.M. of from 4 to 6 individual values.

TABLE 2

THE EFFECT OF EXPOSURE TO DIESEL EXHAUST ON RAT LIVER WEIGHT (GRAMS)*

	Concentration of Diesel Particles		
Duration	Control	250μg/m³	1500μg/m³
6 weeks	7.0 ± 0.2	7.3 ± 0.3	6.9 ± 0.2
12 weeks	7.4 ± 0.2	7.5 ± 0.2	7.1 ± 0.1
24 weeks	7.7 ± 0.2	9.1 ± 0.4	8.3 ± 0.2
53 weeks	9.3 ± 0.6	9.5 ± 0.3	8.7 ± 0.5

*Each value is the mean ± S.E.M. of from 4 to 6 individual values.

indicate that neither exposure affected liver size. However, the effect of the exposure on the activity of hepatic microsomal oxidases responsible for the conversion of benzo[α]pyrene to more polar metabolites (Table 3) was more complicated. Six weeks of exposure to either concentration of diesel particulates appeared to reduce the ability of the hepatic microsomes to oxidize benzo[α]pyrene. At twelve weeks only exposure to the highest concentration interfered with the microsomal oxidation and by six months neither group was significantly different from control. The ability of microsomes to generate metabolites of benzo[α]pyrene which were capable of

TABLE 3

THE EFFECT OF EXPOSURE TO DIESEL EXHAUST ON THE ABILITY OF RAT LIVER MICROSOMES TO OXIDIZE BENZO[α]PYRENE TO POLAR METABOLITES (nmoles per 30 min per mg protein)*

	Concentration of Diesel Particles		
Duration	Control	250μg/m³	1500μg/m³
6 weeks	21.4 ± 0.6	12.5 ± 2.1	10.7 ± 1.0
12 weeks	14.4 ± 1.4	15.7 ± 0.8	8.5 ± 1.3
24 weeks	11.5 ± 0.5	12.3 ± 1.3	12.0 ± 1.1
53 weeks	16.9 ± 2.4	13.6 ± 1.8	12.9 ± 2.7

*Each value is the mean ± S.E.M. of from 4 to 6 individual values.

alkylating DNA was also assayed (Table 4). Again at the briefest exposure interval, the microsomal oxidation seemed to be surpressed, but was restored to normal after longer periods of exposure.

TABLE 4

THE EFFECT OF EXPOSURE TO DIESEL EXHAUST ON THE ABILITY OF RAT LIVER MICROSOMES TO OXIDIZE BENZO[α]PYRENE TO ALKYLATING METABOLITES (nmoles bound to mg DNA per hr per mg protein)*

	Concentration of Diesel Particles		
Duration	Control	250μg/m^3	1500μg/m^3
6 weeks	0.13 ± 0.02	0.04 ± 0.01	0.05 ± 0.01
12 weeks	0.04 ± 0.003	0.04 ± 0.003	0.04 ± 0.004
24 weeks	0.05 ± 0.01	0.05 ± 0.01	0.04 ± 0.01
53 weeks	0.07 ± 0.05	0.08 ± 0.04	0.06 ± 0.01

*Each value is the mean ± S.E.M. of from 4 to 6 individual values.

As might be expected, the exposure to diesel exhaust had more obvious effects on the lungs. As can be seen from Table 5, after a year's exposure to the highest exhaust concentration, the size of a lung pair was about 50% greater than control lungs or lungs from animals exposed to the lowest dose. The most striking effect of the

TABLE 5

THE EFFECT OF EXPOSURE TO DIESEL EXHAUST ON THE WEIGHT OF THE RAT LUNG PAIR (GRAMS)*

	Concentration of Diesel Particles		
Duration	Control	250μg/m^3	1500μg/m^3
6 weeks	1.5 ± 0.1	1.5 ± 0.2	1.0 ± 0.1
12 weeks	1.3 ± 0.1	1.2 ± 0.1	1.5 ± 0.2
24 weeks	1.3 ± 0.1	1.1 ± 0.1	1.6 ± 0.2
53 weeks	1.4 ± 0.2	1.5 ± 0.1	2.1 ± 0.1

*Each value is the mean ± S.E.M. of from 4 to 6 individual values.

exposure was on the appearance of the lungs and tissue fractions prepared from them. The lungs of exposed animals ranged from a gray color after the shortest exposures to a dark gray shot through with dark black deposits after longer exposures, and microsomal membranes prepared from these lungs were contaminated with the dark material so that they were gray to black suspensions instead of the usual pinkish suspension of control microsomes. Neither group exposed to diesel exhaust was able to metabolize benzo[α]-pyrene as well as the controls at any time interval although this effect is most evident after 53 weeks of exposure (Table 6).

TABLE 6

THE EFFECT OF EXPOSURE TO DIESEL EXHAUST
ON THE ABILITY OF RAT LUNG MICROSOMES
TO OXIDIZE BENZO[α]PYRENE TO POLAR
METABOLITES (nmoles per 30 min
per mg protein)*

	Concentration of Diesel Particulates		
Duration	Control	250μg/m^3	1500μg/m^3
6 weeks	0.23 ± 0.08	0.12 ± 0.05	0.16 ± 0.02
12 weeks	0.29 ± 0.02	0.16 ± 0.02	0.23 ± 0.03
24 weeks	0.13 ± 0.05	0.10 ± 0.06	0.07 ± 0.02
53 weeks	0.32 ± 0.05	0.15 ± 0.05	0.02 ± 0.01

*Each value is the mean ± S.E.M. of from 4 to 6 individual values.

By this time the highest exposure had markedly compromized the animals ability to convert benzo[α]pyrene to polar compounds and even exposure to 250 g/M^3 had reduced the enzymatic activity by more than 50%. The way in which diesel exhaust produces this inhibition is not clear. It is possible that the particulates that contaminate the lung microsomes may interfere with the assay of benzo[α]-pyrene metabolism (e.g., reduce the availability of substrate or directly inhibit the microsomal oxidations). Alternatively, the lowered enzymatic activity may reflect a pathological change in the lung endoplasmic reticulum related to the exposure. This remains an open question at present; however we have tried to evaluate the potential of the diesel particulates to inhibit the *in vitro* metabolism by mixing experiments. The inhibitory effects of boiled control microsomes, and boiled low exposure and high exposure microsomes on metabolism of benzo[α]pyrene by untreated control microsomes are compared in the experiment described in Table 7. The results suggest that particulates present in the microsomes prepared from the highest exposure group may inhibit benzo[α]pyrene metabolism; however, these results should be considered preliminary. The production of alkylating metabolites by lung microsomes was too low to be detected by our methods in either controls or exposed animals.

TABLE 7

THE EFFECT OF LUNG TRAPPED DIESEL PARTICULATES ON *IN VITRO* BENZO[α]PYRENE OXIDATION BY LUNG MICROSOMES*

Additions	Percent of Control
None	100
Boiled control microsomes	83 ± 23
Boiled low exposure microsomes	140 ± 16
Boiled high exposure microsomes	65 ± 12

*Microsomes from control lungs were incubated as usual except the indicated additions were made. Boiled microsomes were from a 12 week exposure to clean air, 250 μg per M^3 and 1500 μg per M^3 of diesel particulates. These microsomes were heated at boiling for 5 min in a capped tube and allowed to cool before mg microsomal protein were added to a control incubation. Each value is the mean of five determinations ± S.E.M.

It seems possible to conclude that after slightly over a year's exposure, neither concentration of diesel emissions has had an effect on the growth rate or liver weight of Fischer 344 rats. A reduced capacity for hepatic oxidation of benzo[α]pyrene observed after earlier exposure periods was not evident at later periods, and may either have been repaired or may have been due to an artifact in the experimental procedure. There were, however, more evident changes in both the size of the lungs and in the ability of the pulmonary microsomes to oxidize benzo[α]pyrene. After a year at the highest exposure level lung weight was increased and benzo[α]pyrene metabolism was severely impaired at both exposure levels. The reduced ability to metabolize benzo[α]pyrene was unexpected, and it should be noticed that at no time during these studies was induction of this activity observed.

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General Discussion

B. DANIEL: I noticed that the AHH levels in your control animals dropped after six weeks, is that normal for that species?

R. MCCAULEY: That is right, however, it does seem to be a smooth drop. To answer your question, I would be very tempted to correlate the drop with age, of course, because that is the only thing I would like to believe has changed in the course of these things, but I would be very reluctant to do so.

G. STONER: There is mounting evidence that the metabolism of benzopyrene by microsomal fractions is quite different from that of whole cells. That is, whole cells tend to produce more of the diol compounds as opposed to microsomal fractions. It might be interesting in your studies to compare metabolism of benzopyrene in whole cells and microsomal fractions because there are marked differences.

R. MCCAULEY: That is an interesting notion, but really what we were using this assay for was an indication of the extent of the exposure to potential inducers that might be in the diesel exhaust, fume, particulate, and so the types of metabolites that we are generating really were not the primary interest. In fact, we tried to avoid that whole issue by taking an assay that would extract everything that they made.

E. CANTRELL: We have had some experiences with smoke inhalation. My question is, were your controls just animals from the stock or were they exposed to air?

R. MCCAULEY: The controls were kept in an environmental chamber with the same identical conditions, except no exhaust.

BENZO(A)PYRENE METABOLISM IN MICE EXPOSED TO DIESEL EXHAUST: I. UPTAKE AND DISTRIBUTION

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ABSTRACT

In this study we examined the effect of diesel exhaust (DE) exposure on the disposition of a typical polycyclic aromatic hydrocarbon. DE-exposed and non-exposed A/Jax mice were divided into 3 groups and each mouse instilled intratracheally with benzo(a)pyrene (BP). One group (A) received ^{14}C-BP, and at intervals of 2, 24, and 168 hours, three mice from the group were killed and quick frozen for whole body autoradiography. Saggital sections were cut at 0.5 mm intervals and autoradiograms prepared. Adjacent sections were studied so that radioactive areas were matched to specific organs. The second group (B) received ^{3}H-BP and at 2, 24, and 168 hours mice were killed. Livers, lungs, and testes were weighed and frozen. From these tissues metabolites were analyzed; these data are reported in the next paper.

Histofluorescent examination of tissues from mice instilled with nonradioactive BP (group C) confirmed that BP was present in lung. The autoradiography data is the basis for elucidating the BP distribution in the mouse. Within 2 hours after instillation radioactivity was detected in the entire animals, with most in lungs, liver, and GI tract. By 24 hours after instillation considerable radioactivity had redistributed to the GI tract. At 168 hours after instillation only a trace of label was found in the GI mucosa.

INTRODUCTION

The carcinogenic potential of diesel engine exhaust (DE) can in part be evaluated by considering the fate and distribution of the polynuclear aromatic hydrocarbons (PAHs) in DE on animals exposed to these emissions.

It is believed that the mechanism for carcinogenesis due to PAHs follows the steps shown in Figure 1; similar schemes have been proposed by others (1). Regardless of the exposure modality, the PAH is absorbed and metabolized by some if not all tissues. In the course of enzymatic oxydation of the PAHs, reactive intermediaries are produced which, for the most part, conjugate to more polar (more water soluble) metabolites which are then excreted. However, some reactive intermediaries bind to cellular macromolecules which may produce either cell death or exhibit a potential for tumor transformation. PAH binding to cellular macromolecules has been demonstrated in various proteins, RNA, and DNA. The potential for tumor transformation in cells with DNA-bound PAH derivatives is mitigated by the ability of repair enzymes to delete the damaged portion of DNA. The remaining pathway leads to transformation and tumor formation. However, "immune surveillance" is believed to be active so that tumor cells are recognized as foreign to the body and are killed. At some point in time, either immune surveillance fails or the tumor kinetics overwhelm the immune system. This results in a tumor.

An understanding of fate and distribution of PAH metabolites can lead to at least a partial understanding of the carcinogenic potential of specific PAHs within the context of exposure conditions. We evaluate here the distribution of benzo(a)pyrene (BP) given intratracheally to mice which were either exposed or not exposed to Diesel exhaust. This paper presents the data on the distribution of BP by examining autoradiograms made of whole body sections obtained from mice. The following paper analyzes more quantitatively the fate of BP by analyzing the time course of clearance and metabolite formation in three critical tissues, liver, lung and testes. Analogous fluorometric data will be reported later.

MATERIALS AND METHODS

Diesel Exhaust Exposure

Emission source. The diesel exhaust was generated using a Nissan CN6-33 diesel 6 cylinder engine and a Chrysler torqueflite automatic transmission Model A-727 coupled to an Eaton-Dynamometer Model 758-DG. The engine was cycled using a repetitive series of nine driving modes known as the Federal

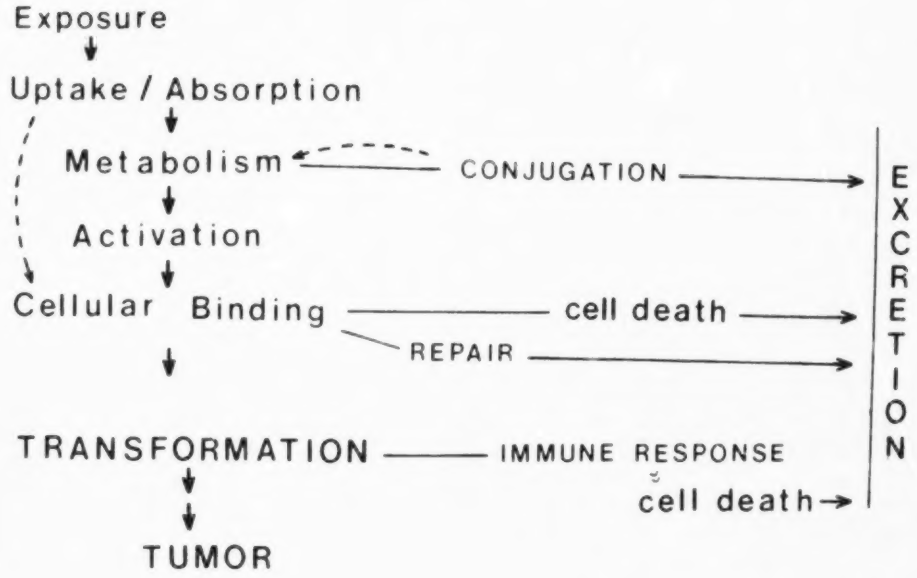

Figure 1. Carcinogenesis pathway for polynuclear aromatic hydrocarbons.

Short Cycle, used in the present fuel emission studies. Continuous cyclic monitoring of the chamber atmospheres were carried out for carbon dioxide, carbon monoxide, total hydrocarbons, sulfur oxides, nitric oxides, and nitrogen oxides, and particulates were collected daily. Further details are available (2).

Animal exposure. The animals used in this study were weanling male Strain A/J mice that were housed and exposed at the USEPA, Center Hill Facility in Cincinnati. The diesel-challenged mice were exposed to raw diesel exhaust which was diluted with filtered conditioned air at a 16:1 to 18:1 dilution ratio, to achieve a 6 mg/m^3 of particulates atmosphere. Exposure time was eight hours per day, seven days per week, for nine months. All mice were maintained in stainless steel exposure chambers. The control mice were housed in identical chambers under identical conditions, except filtered, conditioned air was substituted for the diluted diesel exhaust. Mice were fed Purina Rat Chow and tap water ad libitum.

Animal Preparation

Instillation. Prior to instillation the animals were anesthesized. To reduce mucous secretions 0.1 ml (1 mg/kg) atropine was injected i.p. Approximately 1 minute later the animals were injected i.p. with 0.25 ml Pentobarbital (40 mg/kg).

After anesthetization, the animals were instilled with BP using a restraining device, a modified surgical otoscope and a beaded 22-gauge lumbar needle. The restraining device was used to support the animal, hold its mouth open, and to hold the trachea in a straight line. The otoscope speculum was modified so that its outer diameter was approximately 2 mm. A 22-gauge needle was modified by first removing the bevel tip and then placing a bead of epoxy plastic near the end. The dispenser was a Hamilton repeating dispenser with a 1 ml reservoir and dispensing 20 microliters per increment. After placing the animal in the restraining device, the otoscope was used to visualize the vocal cords, and the needle was placed through the speculum, between the cords and into the trachea. A volume of 20 microliters gelatin solution containing BP in suspension was deposited. The solution consisted of 0.2% gelatin and 0.05 micrograms/microliter of BP. Each dose contained 1.0 μg of BP which was either nonradioactive or labeled with ^{14}C (0.05 μCi). Each animal was observed for respiratory distress until recovery from anesthesia.

Autoradiography

Mice were sacrificed by cervical dislocation at approximately 2 hours, 24 hours, and 168 hours after instillation with ^{14}C-BP. Immediately thereafter each mouse was placed into a 50 ml plastic centrifuge tube and immersed for approximately five minutes in liquid nitrogen. Upon removal, each mouse was placed in a cooled polyethylene zip-lock bag and placed in dry ice and later transferred to a freezer maintained at -20°C. Autoradiographic procedures were based on the method of Ullberg (3) and are detailed below.

Specimen mounting. Animals instilled with ^{14}C-Benzo(a)pyrene were received in the frozen state and were maintained at -15°C until processed. The animals were quickly scrubbed with a 1.3% solution of sodium carboxymethylcellulose (CMC) (Apoteket Marden, Stockholm) in distilled water, taking care to wet the fur. Using a removable mounting frame, a 2 cm layer of 1.3% CMC was frozen on a 1-1/2" x 4-5/8" brass mounting base by immersion in liquid nitrogen. The mouse was placed on its side on the frozen layer and covered to a depth of approximately 1 cm with the 1.3% CMC solution and quickly immersed in liquid nitrogen, being careful to prevent the top central portion from solidifying until the entire block was almost solid. The mounting frame was removed, and the block stored for a minimum of 24 hours at -12°C before cutting.

Specimen sectioning. The blocked animals were positioned in the jaws of a Leitz Model 1300 sleigh microtome (E. Leitz, Wetzlar, Germany) which was modified to be hydraulically driven at 7.5 cm/sec and installed in a custom-built -12°C cryostat. The entire animal was cut into 50 micron saggital sections, labeling and transferring every alternate section on clear tape (Scotch Brand Prescription Label Tape #800). Every 20th section was placed on an open frame for freeze drying; all other sections were stored on waxed paper for future reference. An average of 906 sections were made per mouse with sections for autoradiography each half millimeter. Microtome blades were exchanged for every mouse and were sharpened by the Weck Corporation, Research Triangle Park, NC. The frame-mounted sections were held in a freezer at -15°C for a minimum of 24 hours to allow for freeze drying.

Section exposure. When the outer CMC portions of the section could be handled without melting, the 1-1/2" x 4-5/8" sections were transferred in total darkness to 5 x 7" Kodak No-Screen (#NS-2T) Ready Pack film (Eastman Kodak, Rochester, NY), two sections per film. The films with sections attached were returned to their folders and stacked in groups of 12 with standard weight blotter paper separating each film to a thickness of approximately 1" and pressed between two 1/8" aluminum frames held with four #100 binder clips (IDL Mfg.

Co., Carlstadt, NJ). The package was double-wrapped in aluminum foil and stored for 28 days at -15°C for exposure. Exposure controls indicated that 28 days were adequate, with no further resolution being obtained at up to 56 days. Exposed films were processed in total darkness at 20°C for five minutes in Kodak Liquid X-Ray Developer & Replenisher (#146 5335, Eastman Kodak, Rochester, NY), and 10 minutes in Kodak Rapid Fixer (#146 4114, Eastman Kodak, Rochester, NY). Developer and Fixer were replenished after each group of 12 films and were discarded after processing 48 films. Films were rinsed in running tap water for 20 minutes and allowed to air dry. The entire process was conducted with appropriate radiation safety measures.

Histofluorescence

Mice of group C were anesthetized and each was injected with 1.0 ml of filter-sterilized bacterial protease (2 mg) via the tail vein. After one minute the mouse was sacrificed by cervical dislocation, and the liver, lung, and testes removed and weighed. Half of each organ was immediately frozen for later histologic studies, the other half was prepared for other analyses.

Microtomy. The frozen organ aliquots were stored at -90°C until the day of microtomy. Frozen sections were prepared under reduced light and the sections placed on glass slides while remaining frozen. Multiple slides were prepared for each organ. Just prior to examination by fluorescence microscopy, a slide was thawed and air dried. For visualization of BP and the collective group of intracellular metabolites, the sections were examined by fluorescence microscopy with excitation provided by a Xenon lamp filtered through a UG 1 and a BG 12 Corning filter, on a epi-illumination mode. Fluorescence above 400 nm was visualized. For selected tissues, photographs were prepared.

RESULTS AND DISCUSSION

The characteristic blue fluorescence of BP was observed in the lung bronchi, extending into the lobes. This confirmed that the BP was deposited in the bronchi. The distribution of BP throughout the tissues could not be observed because of the low level and diffuse character of the fluorescence. Figure 2 shows a 100X magnification of the lung section from a nonexposed mouse which was sacrificed two hours after instillation. The bright areas are the BP taken up by the tissues with the brightest foci corresponding to unabsorbed BP crystals. The three dark areas are the bronchiole which were slightly opened by the happenstance of sectioning the tissue at a fortunate location.

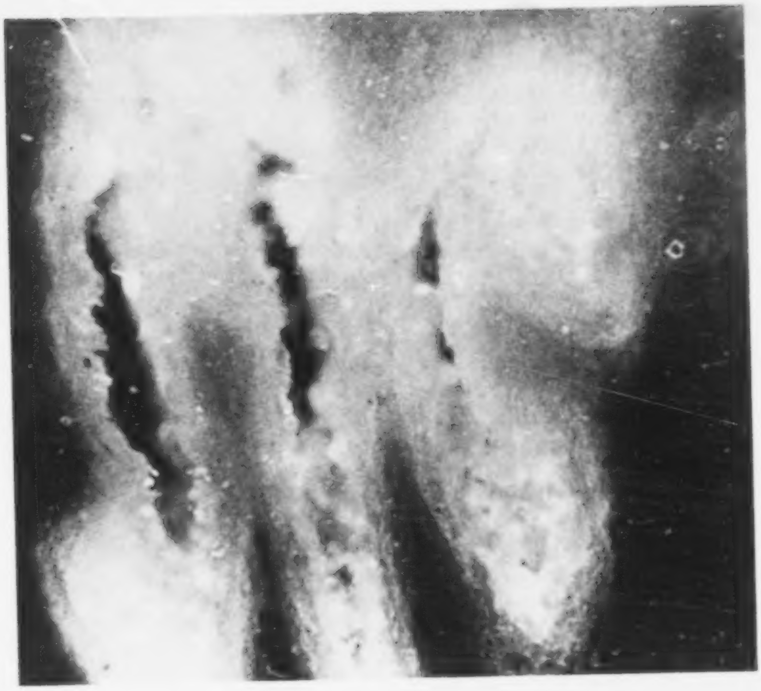

Figure 2. Histofluorescence of lung section. Photomicrography of Benzo(a)pyrene fluorescence from the bronchiolar lining.

Autoradiography was performed on DE-exposed or non-exposed mice which were killed at 2, 24, and 168 hours after instillation with ^{14}C-BP. Each group was done in triplicate. Ten sections are shown from each of two DE-exposed mice sacrificed at 2- and 24-hr time points (Fig. 3 and Fig. 4, respectively). Autoradiograms of 168-hour exposed mice (not presented) revealed no radiographic exposure except for a consistent image of the stomach.

Figure 3 is a sequence of autoradiograms made from sections taken at the indicated distance in millimeters from the skin on the left side of the mouse. The head is to the left. From the original autoradiograms, compared with adjacent tissue sections as well as anatomical analysis of intact mice, the following is apparent. The mice received an intratracheally instilled bolus of radioactive material. In some cases the animal would pass part of the bolus to the oral-nasal cavity and return a portion of the bolus through the esophagus to the stomach. Animals that had been sacrificed within 2 hours of instillation showed substantial radiographic exposure of the entire tissue section indicating the ^{14}C-BP had been passed to the blood stream and circulated throughout the whole animal. Specks of exposure can be seen in the regions corresponding to the lung, further confirming the placement of the ^{14}C-BP in this organ. The images of the kidneys and bladder are quite prominent which indicates a rapid accumulation of the ^{14}C-BP in the urine. More than 10% of the total radioactivity given to the animal had been excreted in the urine within 16 hours of instillation. Images of the liver and GI tract are also quite prominent at this time, indicating a rapid uptake of the ^{14}C-BP from the stomach and portal blood. At no time did we see the reproductive organs outlined.

Figure 4 is a similar montage of autoradiograms of tissue sections. However, sectioning was from right to left, the head is on the right side and the mouse had been sacrificed 24 hours after instillation. By this time visualization of the entire animal was no longer possible. Imaged organs were associated with 1) GI tract, liver and stomach on down; 2) urinary tract, kidneys and bladder; and 3) a faint image of the lung. Highly prominent are the images of the fecal material in the intestines and the speckled character of the bladder image. Not presented are the autoradiographic images of the mice sacrificed 168 hours after instillation. These showed images corresponding to the rugae of the stomach. Apparently, the stomach lining retains large amounts of the radioactive hydrocarbons after other tissues have been cleared sufficiently to prevent imaging.

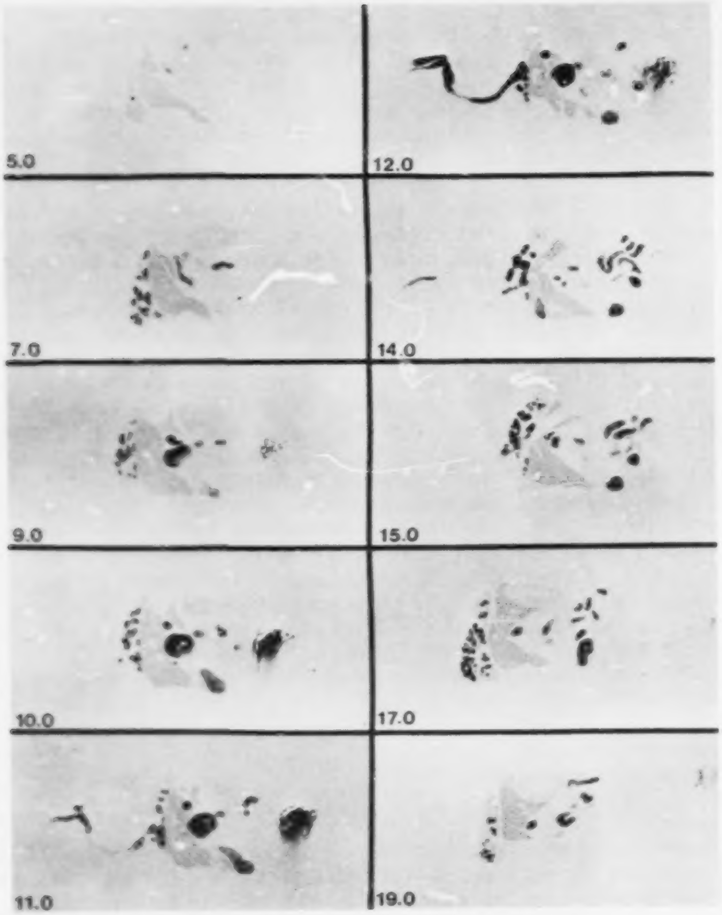

Figure 3. Photographs of autoradiograms made from 10 tissue sections of a mouse. Benzo(a)pyrene instillation was 2 hours prior to sacrifice. Sections were cut from the left side and the numbers are the distances from the first cut to the section, in millimeters. The head is to the left.

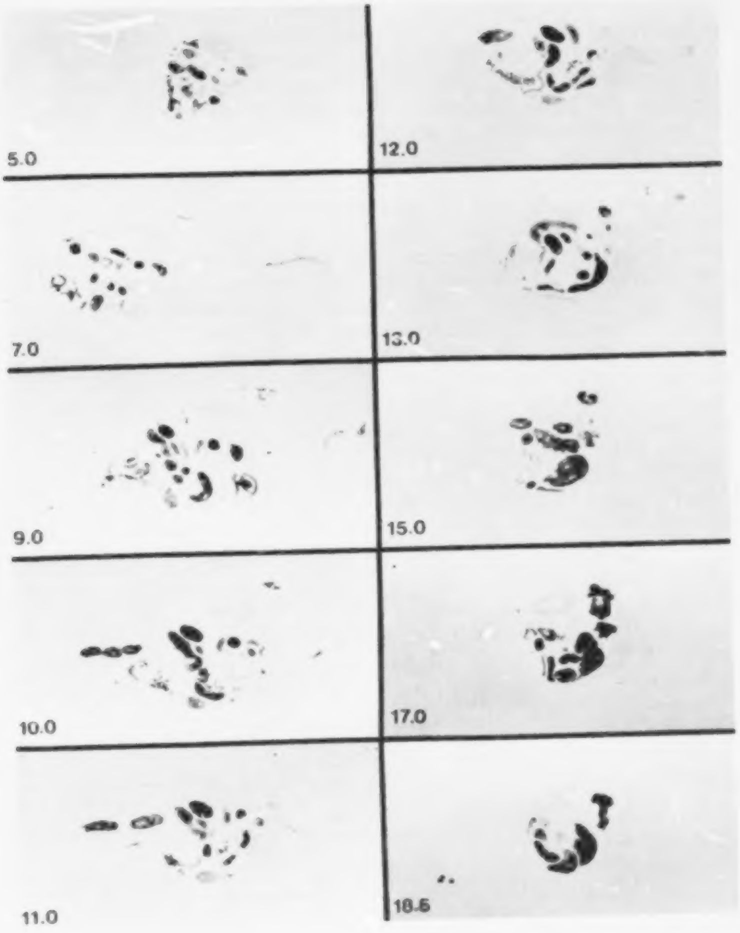

Figure 4. Photographs of autoradiograms made from 10 tissue sections of a mouse. Benzo(a)pyrene instillation was 24 hours prior to sacrifice. Sections were cut from the right side and the numbers are the distance from the first cut to the section, in millimeters. The head is to the right.

CONCLUSIONS

Qualitatively there are no obvious differences between the autoradiograms of the DE exposed mice and those that had not been so exposed. Far greater differences occurred between mice with the same treatment, presumably reflecting their ability to expectorate a portion of the radioactive bolus.

Figure 5 summarizes the distribution of ^{14}C-BP in both DE-exposed and non-exposed mice. Briefly, the ^{14}C-BP is placed in the lungs. Within two hours it is circulating in the blood stream and may have entered the esophagus and stomach by what amounts to coughing and swallowing. From the blood ^{14}C-BP is accumulated in the kidneys and bladder then excreted. From the blood stream and digestive tract ^{14}C-BP enters the liver. By 24 hours, accumulation occurs in the lower GI tract, the liver and stomach, the kidneys and bladder, as well as a slight amount in the lungs. By one week some BP is found in the stomach.

Quantitative considerations are more sensitive in differentiating DE-exposed from unexposed mice. Such an analysis is reported in the next paper. It is apparent from these studies that the metabolism of BP is more affected by DE exposure than its distribution.

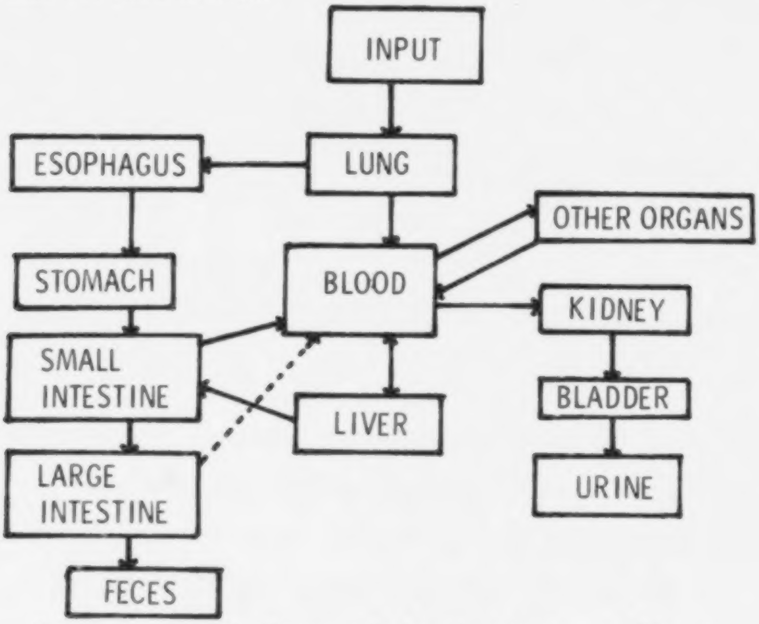

Figure 5. A block diagram of the distribution of ^{14}C-BP in mice.

ACKNOWLEDGMENTS

This study supported in part by EPA contract #68-03-2790, NIH grant #CA-HL-15784, and grant #1120 from the Council for Tobacco Research-U.S.A., Inc. The authors are also grateful for the technical assistance of C. Hernandez, H. Jones, L. Wilson, P. Eaton, and L. McMillan.

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BENZO(A)PYRENE METABOLISM IN MICE EXPOSED TO DIESEL EXHAUST: II. METABOLISM AND EXCRETION

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ABSTRACT

In this study we examined the effect of diesel exhaust (DE) exposure on IN VIVO metabolism of benzo(a)pyrene (BP). DE-exposed and un-exposed A/Jax mice of group B were instilled intratracheally with ^{3}H-BP. At each time point of 2, 24, and 168 hours after instillation 5 mice were killed and the liver, lungs, and testes were removed and frozen. Aliquots of the organs were homogenized in 2 ml water and each received 3 volumes of cold ethanol. Radioactivity in supernatant and precipitate was measured. The supernatant extracts were subjected to HPLC analysis on ALOX-T and on Zorbax ODS. The ALOX-T method was a modification of Autrup's procedure for conjugate assay (Biochem. Pharmacol. 28:1727, 1979). Fractions were: a) free BP; b) nonconjugated primary metabolites; c) sulfate conjugates; d) glucuronides, glutathiones and other conjugates. By 2 hours after instillation primary metabolites were found in liver and lung, but very little was conjugated. The unconjugated BP was mainly in the form of free BP and phenolic metabolite(s). The lungs of DE-exposed mice had less capacity to dispose of "bound" BP 1 week after instillation.

INTRODUCTION

Particulate organic matter in diesel exhaust is known to contain approximately 1% polynuclear aromatic hydrocarbons (PAHs) by weight. Diesel exhaust particulate matter may be separated into acid, base, and neutral fractions: the neutral fraction which contains PAHs shows the highest mutagenic effects of the three fractions and each fraction is directly mutagenic, exhibiting frameshift activity (1). Because of the economically attractive future for diesel power and its carcinogenic potential, it is necessary to test diesel exhaust for its effect on the monooxigenase enzyme system. This enzyme system has been found to convert several polynuclear aromatic hydrocarbons into more reactive intermediaries which bind to cell macromolecules including DNA, RNA, and proteins (2). Such binding may lead to cancer (3,4).

In this study, HPLC analysis was used to determine the BP conjugates and to quantitate the disposition of the BP. Additionally, HPLC was used in a reverse phase mode to identify the organic soluble metabolites of BP. The ^{3}H-BP which was not extractable with a polar solvent gave an estimate of tightly bound BP in the tissues.

MATERIALS AND METHODS

Animals, exposures and instillation procedures have been detailed in the preceding paper.

After instillation with 5 μCi ^{3}H-benzo(a)pyrene (BP), animals were sacrificed by cervical dislocation at 2 hours, 24 hours, and 168 hours. The liver, lungs, and testes were removed at once, weighed and frozen on dry ice. The organs were kept frozen until homogenized. Organs were removed from the freezer and cut into halves. One half was returned to the freezer and the other half weighed and thawed. Each organ aliquot was placed into 2.0 ml water and homogenized in a glass-glass tissue grinder. Three volumes of ice-cold ethanol was added to each homogenate and allowed to stand at least 2 hours in the refrigerator. The precipitates were sedimented by centrifugation for 15 minutes at 50,000 x g. The aqueous ethanol supernatant of each homogenate received 5 μl of Vitamin E to serve as antioxidant and was then dried under a nitrogen stream. The residue was redissolved in 1.2 ml 80% ethanol and half was injected onto an HPLC column. Ten μl aliquots of the remainder was taken for scintillation counting. The pellet fractions mentioned above were suspended in 2.0 ml H_2O and 50 μl taken for scintillation counting. The HPLC column was 4.6 x 100 mm, packed with neutral alumina. The bed was previously perfused with 0.5 M phosphate buffer, pH 7.0, followed by water, ethanol, then hexane. The 200 μl injection was made during solvent flow of 2.0 ml/min. BP was eluted with 10 ml hexane; hydroxy BP was eluted with 15 ml ethanol; BP-sulfate was eluted with 15 ml water; unknown material eluted with 15 ml 0.5 M phosphate, pH 3.0; BP-glucuronide and BP-glutathione eluted with 20 ml 25% formic acid.

The column was restored by washing with neutral phosphate buffer, water, ethanol, and hexane. The identification of the metabolites in each fraction was confirmed by elution of authentic standards. The glutathione conjugates were presumed to be eluted by formic acid (5). Radioactivity in each fraction was determined in a Beckman LS7000 liquid scintillation counter.

For selected samples, the hexane plus ethanol fractions were dried and the residue redissolved in 0.25 ml ethanol. A part (0.10 ml) was injected into a Dupont Zorbax ODS column and eluted with a linear 50-100% methanol in water gradient. The flow rate was 1.6 ml per minute and 0.8 ml fractions were collected in mini-vials. To each vial was added 5 ml Scintiverse and each vial was counted 10 minutes. The profile of nonconjugated metabolites was plotted.

The urine of each group of mice was collected over the first 16 hours after instillation and the radioactivity determined. A sample of caecum feces was taken from one mouse sacrificed 24 hours after instillation and the conjugate profile prepared.

RESULTS AND DISCUSSION

The time course of disappearance from lung, liver, and testes is illustrated in Figure 1. There was no difference in clearance of soluble metabolites by the DE and control groups. Both groups were capable of clearing the bound BP, but there was a significantly higher amount of residual BP in lungs of DE mice one week after instillation.

Metabolism of BP can occur in all three of the tissues we examined (6). This notion is supported by the presence of both primary and secondary metabolites in the tissues at the times after instillation. Figure 2 presents a summary of the recovery of BP and its metabolites within two hours after instillation. The total radioactivity in the alcoholic extract represents unity on the ordinate. The bars represent the proportional amount of each fraction. In lung, the majority of radioactivity was as nonmetabolized BP. In contrast, liver and testes contained more than half of the radioactivity as metabolites with a substantial degree of secondary metabolism to conjugates. The DE animals appeared to have less free BP in tissues and this may reflect on induction of the enzymes for primary metabolism of polycyclic aromatic hydrocarbons. Within 24 hours after instillation the BP in these tissues was principally in the form of metabolites, with a substantial degree of conjugation (Fig. 3). Likewise, the BP was present mainly as metabolite by 168 hours after instillation (Fig. 4). Given the low total radioactivity as illustrated in Figure 1, the metabolite

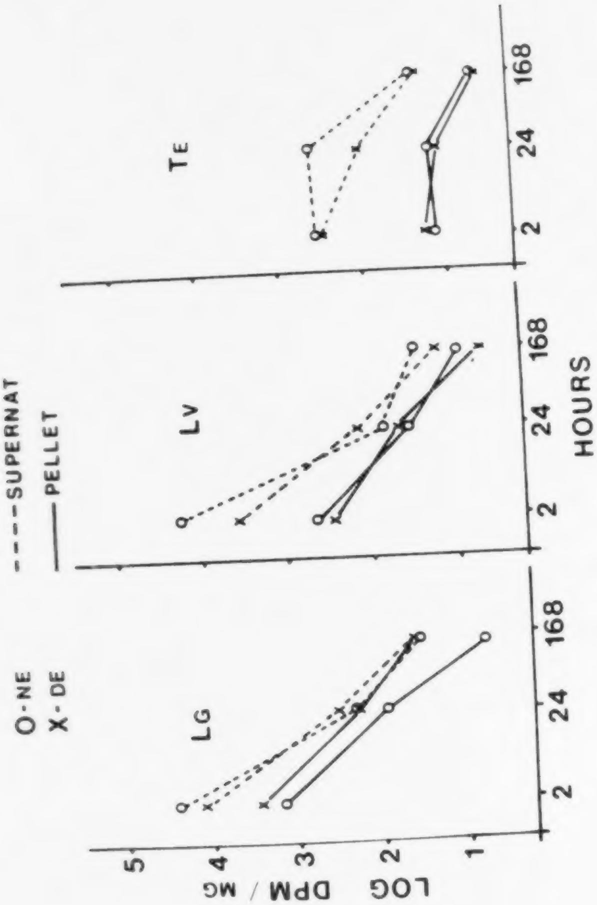

Figure 1. Disappearance of radioactivity after instillation. NE, non-exposed; DE, diesel-exposed; Lg, lung; Lv, liver; Te, testes. Each point represents the mean of 5 mice. Radioactivity is expressed as DPM per mg wet tissue.

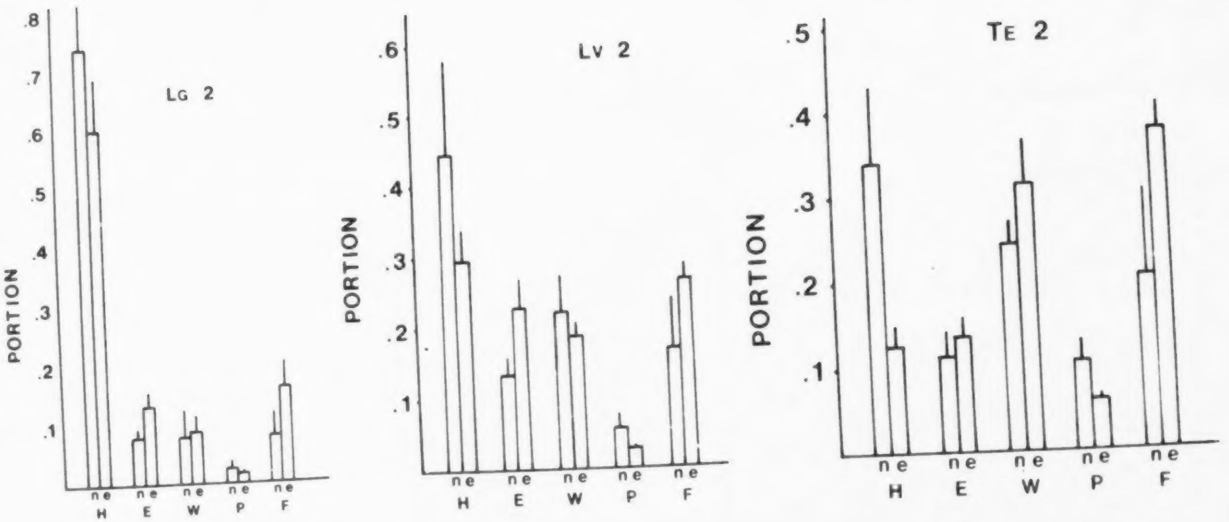

Figure 2. Relative amount of metabolites and free BP in 3 tissues two hours after instillation. H, hexane fraction containing BP; E, ethanol fraction containing hydroxy metabolites; W, water fraction containing sulfate conjugates; P, phosphate fraction; F, formate fraction containing glucuronides and glutathione conjugates. n, non-exposed; e, diesel-exposed. The vertical lines represent the S.E.M.

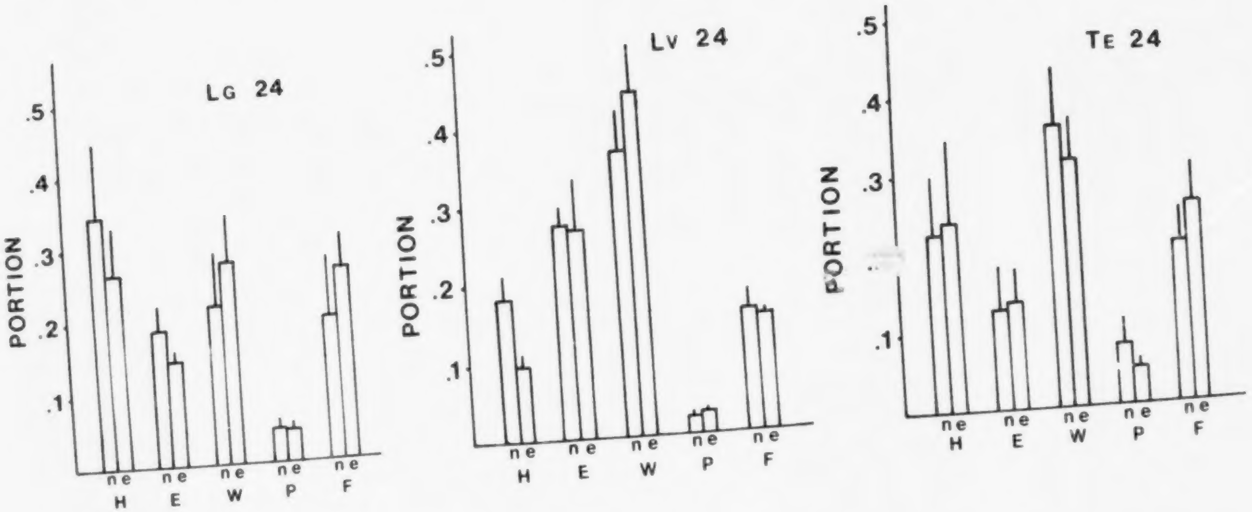

Figure 3. Relative amount of metabolites and free BP in three tissues 24 hours after instillation.

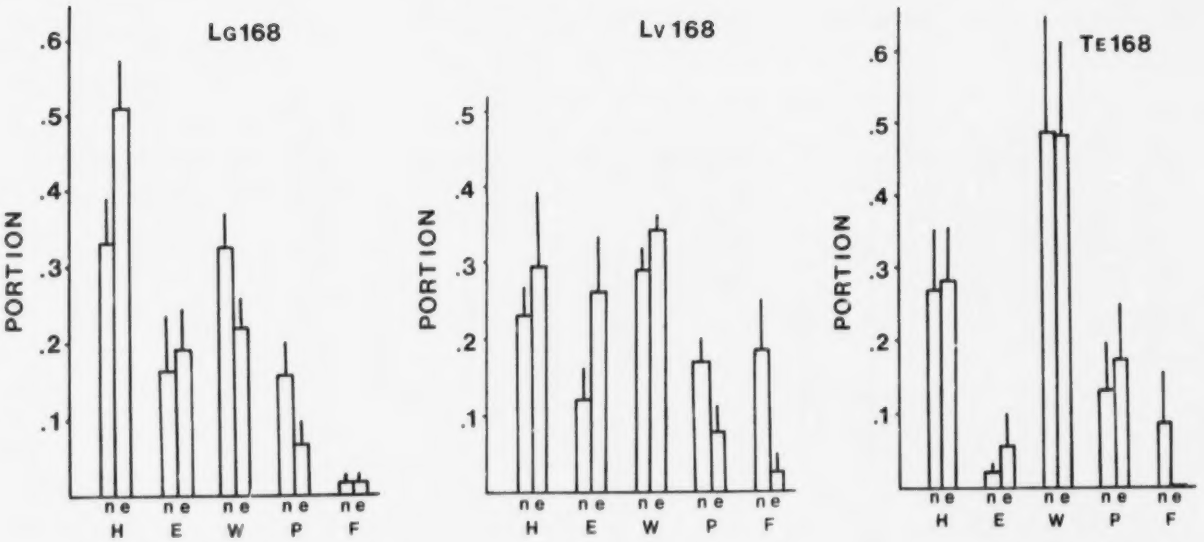

Figure 4. Relative amount of metabolites and free BP in three tissues 168 hours after instillation.

profile may not be meaningful for liver and testes. The high total amount and proportion of unmetabolized BP in DE lung at 168 hours suggests that a small amount of BP is adsorbed to smoke particles. These have a prolonged sojourn in the body (7) and may provide a means whereby carcinogenic hydrocarbons may be very slowly cleared at low levels.

The predominate primary metabolite of BP in liver was 3-hydroxy-BP. This was evidenced by the reverse phase HPLC profile illustrated in Figure 5. This does not indicate that other metabolites formed, but that the 3-hydroxy-BP is more readily retained in the cells (8,9).

In the preceding paper we had seen that there was a substantial amount of radioactivity in the large bowel and in the GI tract. We were interested in determining whether the radioactivity was benzopyrene that had been swallowed and never absorbed, or whether we were dealing with enterohepatic recycling of conjugates. The contents of the caecum in a 24 hour animal contained some free benzopyrene. but we found a large amount of primary metabolites and very little conjugates (Fig. 6). This is consistent with the concept of hydrolysis of conjugates in the large bowel by the bacterial flora. An alternative explanation is that these metabolites are a result of the mucosal cells in the small intestine metabolizing the benzopyrene and then excreting the primary metabolites right back into the lumen.

The finding of rapid metabolism of the administered BP and accumulation in the GI tract suggests that clearance of a total body load is quite efficient in both groups of mice. Indeed, even the kidneys contributed substantially to the excretion capacity. During the first 16 hours after instillation 18% of the BP was excreted in urine by non-exposed mice and 14% of the BP was excreted in urine by DE mice.

The only meaningful difference we found in DE mice was the suppressed ability to clear the small amount of BP. We suggest that this BP is adsorbed to smoke particles. The implications of this finding are more profound when one considers the probability of human subjects combining cigarette smoking with fuel smoke exposure. The extension of time carcinogens spend in the lung will markedly increase the carcinogenic risk.

ACKNOWLEDGMENTS

This study supported in part by EPA contract #68-03-2790, NIH grant #CA-HL-15784, and grant #1120 from the Council for Tobacco Research-U.S.A., Inc. The authors are also grateful for the technical assistance of A. Loudin, L. Oborn, L. McMillan, L. Wilson, and P. Eaton.

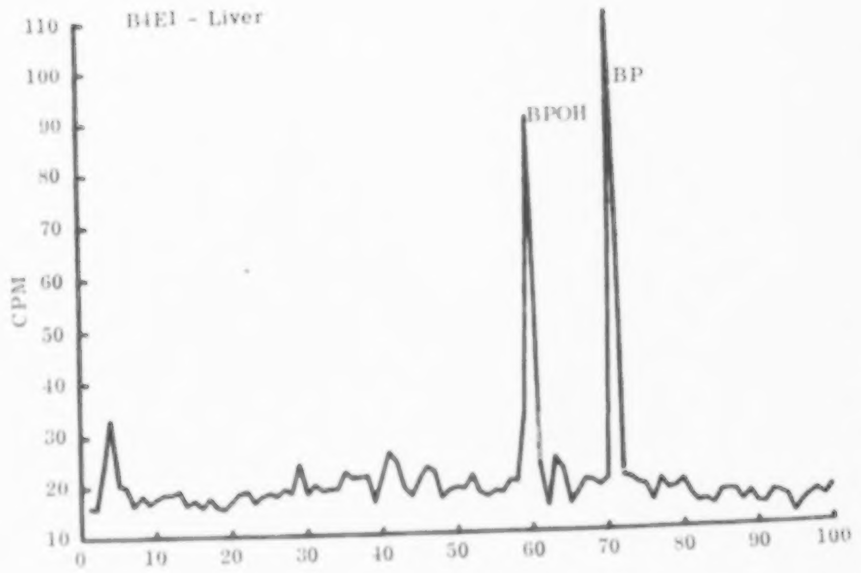

Figure 5. Reverse phase HPLC profile of H+E fractions obtained from a mouse liver extract. The alcoholic tissue extract is from a DE mouse 24 hours after instillation. The abcissa is fraction number and the ordinate is CPM in each fraction. The two major peaks are benzo(a)pyrene and 3-OH-benzo(a)pyrene.

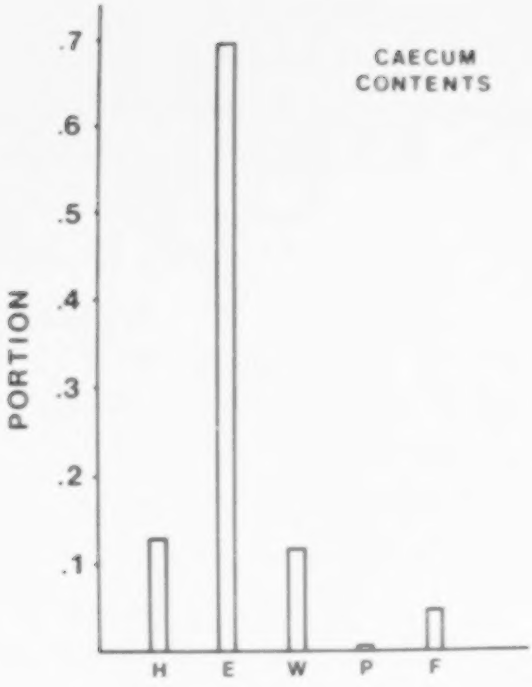

Figure 6. Relative amount of metabolites and free BP in feces from a mouse taken 24 hours after instillation. See legend of Figure 2 for details.

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EFFECT OF EXPOSURE TO DIESEL EXHAUST ON PULMONARY PROSTAGLANDIN DEHYDROGENASE (PGDH) ACTIVITY

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ABSTRACT

There has been evidence that an acute exposure of laboratory animals to nitrogen dioxide (NO_2) for a short period of time can cause marked inhibition of pulmonary PGDH activity. Since diesel exhaust contains NO_2, we have undertaken this investigation to determine the effect of long-term exposure of guinea pigs and rats to diesel exhaust. The present study involves measurement of PGDH activity in the lung tissue as obtained from these animals following exposure to 250 and 1500 μg particulates/m^3 for various time periods in relation to the appropriate time-matched controls. In guinea pigs, the exposure of 1.5 months of low dose of diesel exhaust seems to stimulate the PGDH activity about two-fold while the exposure to higher concentrations of diesel exhaust for 3 months, as well as 6 months, seem to show concentration dependent lowering of PGDH activity as compared to the time-matched controls. This study also documents the well-known species difference in PGDH activity in that the rat shows much lower activity of this enzyme and therefore this species is not suitable for determination of the effect of diesel exposure on the PGDH activity.

INTRODUCTION

15-Hydroxy prostaglandin dehydrogenase (PGDH) oxidizes the 15-OH group of naturally occurring prostaglandins (PGs) to their corresponding 15-keto form (1). Like cyclic nucleotide phosphodiesterase, acetylcholinesterase and monamine oxidase, PGDH is among the group of regulatory enzymes inactivating compounds of high biological potency. Therefore, it has been considered as one of the crucial factors governing the physiological and pharmacological concentrations and actions of PGs.

Furthermore, it has been recently shown that pulmonary PGDH activity can be altered by an acute exposure of guinea pigs to nitrogen dioxide (NO_2) for a short period of time (2). Since diesel exhaust contains NO_2, we investigated the effect of long-term exposure of guinea pigs and rats to diesel exhaust on pulmonary PGDH activity.

EXPERIMENTAL PROCEDURES

MATERIALS USED

(9-^{3}H) Prostaglandin $F_{2\alpha}$ (9.2 Ci/mmol) was purchased from New England Nuclear Corporation, Boston, MA. Prostaglandin $F_{2\alpha}$ THAM was a generous gift of Dr. J.E. Pike, the Upjohn Company, Kalamazoo, MI. Nicotinamide adenine nucleotide was obtained from Sigma Chemical Company, St. Louis, MO. TLC plates (plastic), coated with silica gel and liquid scintillation cocktail, Redi-solv EP were obtained from Eastman Kodak Company, Rochester, N.Y. and Beckman Instruments, Inc., Fullerton, CA., respectively. Guinea pigs (Hartley) and rats (Fischer 344) were supplied by Charles River Breeding Labs., Inc., Wilmington, MA.

Method of Exposure of Animals to Diesel Exhaust

Guinea pigs and rats were exposed to diesel exhaust for various lengths of time at the General Motors Technical Center, Warren, Mich (3). Two doses, namely 250 and 1500 µg particulates/m^3 were used and the control animals were maintained under similar conditions, breathing clean air.

Method of Assay of Prostaglandin Dehydrogenase Activity

The method of assay used was essentially that of Chaudhari *et al*. (2). The reaction mixture consisted of 0.25 ml of 0.25 M sodium phosphate buffer, pH 8.0; 0.05 ml of $PGF_{2\alpha}$ THAM (1 mg/ml), 0.2 ml of cytosol (100,000 x g supernate) fraction of lung homogenate containing 1 mg protein, and water to make up the volume to 1 ml. The mixture was incubated for 15 min at 37° C and the reaction was stopped by acidifying the solution to pH 3.5 with 0.1 N HCl. Eight ml of ethyl acetate was used to extract the substrate and metabolite. The organic phase was evaporated to dryness and the residue was dissolved in 100 µl of absolute methanol. An aliquot of 40 µl was spotted on the TLC plate and the chromatogram was developed in a mixture of acetone:methylene chloride:acetic acid (40:60:1.5 v/v). After drying the chromatogram with a hair dryer, 0.5 cm segments of the chromatogram were cut and counted in a Beckman liquid scintillation spectrometer using Redi-solv EP cocktail.

RESULTS AND DISCUSSION

Table 1 shows the results from the study on the effect of 6 months of exposure of guinea pigs to two doses of diesel exhaust on pulmonary PGDH activity. The control group of animals showed an age-dependent increase in the PGDH activity. The guinea pigs exposed to 250 µg particulate/m^3 (low dose) showed, in comparison to their time-matched control, an initial stimulation of the enzyme activity by about two-fold after 1.5 months, while, paradoxically, the animals exposed to 1500 µg particulate/m^3 (high dose) did not show any change in the PDGH activity. Three months exposure of the guinea pigs to low dose of diesel exhaust did not have any effect on the enzyme activity, but exposure to high doses for the same length of time produced an inhibition of about 26% of PGDH activity as compared to control. Furthermore, it appears that exposure of guinea pigs to low dose for 6 months has lowered the enzyme activity but this difference is statistically not significant. Exposure of guinea pigs to high dose of diesel exhaust for 6 months markedly affected the PGDH activity in lung, producing a significant inhibition of about 43% in comparison to control.

TABLE 1. EFFECT OF EXPOSURE OF GUINEA PIGS TO DIESEL EXHAUST ON PULMONARY PROSTAGLANDIN DEHYDROGENASE ACTIVITY

Time Following the start of exposure (months)	PGDH Activity, nmoles 15-keto PGF$_{2\alpha}$ formed/min/mg protein		
	Time-matched Control[a]	Exposed to 250 µg particulate/m^3	Exposed to 1500 µg particulate/m^3
1.5	0.458 ± 0.059	0.881 ± 0.116[b]	0.469 ± 0.054
3	0.631 ± 0.053	0.514 ± 0.032[c]	0.464 ± 0.044[b]
6	0.757 ± 0.030	0.574 ± 0.117[c]	0.428 ± 0.052[b]

[a] Control animals (n=6) were exposed to clean-air under similar conditions.

[b] Significantly different from control, $p < 0.05$ by Student's t-test, $n = 6$.

[c] Not different from control, $p > 0.1$ by Student's t-test, $n = 6$.

It is speculated that this time-dependent inhibition of PGDH activity as exhibited by the exposure of guinea pig to the high dose of diesel exhaust may be related to various oxidant gases present in the diesel exhaust. Recently, Chaudhari et al. (2) have observed a time-dependent inhibition of PGDH activity in guinea pig due to exposure to 46 ppm NO_2, which reached its maximum inhibitory level after 8 hours of exposure.

Although the high dose of diesel exhaust used in the present study contains a very low level of NO_2, approximately 6 ppm (3), it is possible that an exposure for 3-6 months to the low level of NO_2 can produce similar inhibitory effect on PGDH activity as that of 46 ppm NO_2 for a short exposure of 8 hours. In the context of this probable cause and effect relationship between NO_2 and inhibition of PCDH activity, it is of particular interest to note that the stimulation of PGDH activity observed during exposure to a lower dose of diesel for a shorter period of time may have some parallelism to initial stimulation of the enzyme observed by Chaudhari et al. (2) after exposure to 46 ppm for 1 hour. Presently, additional studies are being done to determine whether the mathematical product of the concentration of NO_2 and the duration of exposure is responsible for the biphasic effect on this enzyme.

At this time, it is not known, either from the present study or from the previous study of Chaudhari et al. (2), the mechanism by which NO_2 affects this vital enzyme or whether the inhibition of this enzyme leads to elevated prostaglandin concentrations in the lung. However, it has been reported by Chaudhari et al. (2) that exposure of guinea pig to 100% O_2 for 48 hours also inhibits pulmonary PGDH activity. Kinetic analysis of the enzyme inhibition by O_2 showed a non-competitive effect with respect to both prostaglandin $F_{2\alpha}$ and NAD^+ which indicates a lack of availability of catalytic sites. Being an oxidant gas, it is possible that NO_2, like O_2, affects PGDH activity by some oxidative mechanism.

The results obtained from exposure of rats to diesel exhaust on pulmonary prostaglandin dehydrogenase activity are summarized in Table 2, where the same enzyme assay system was used as that of guinea pigs. As it is evident from the table, most of the samples did not show detectable amounts of PGDH activity in Fischer Rats 344 from both control and exposed groups. Because of this problem, a proper evaluation of the effects of diesel particulate exposure could not be made in this species.

TABLE 2. EFFECT OF EXPOSURE OF RATS TO DIESEL EXHAUST ON PULMONARY PROSTAGLANDIN DEHYDROGENASE ACTIVITY

Time Following the start of exposure (months)	PGDH Activity, nmoles 15-keto $PGF_{2\alpha}$ formed/min/mg protein		
	Time-matched Control[a] (n)	Exposed to 250 μg particulate/m^3 (n)	Exposed to 1500 μg particulate/m^3 (n)
1.5	0.055 (2)	0.045 (2)	0.076 (1)
3	0.022 ± 0.002 (5)	0.019 (1)	0.019 ± 0.002 (3)
6	0.019 (1)	0.014 (1)	0.030 ± 0.008 (3)

[a] Control animals were exposed to clean-air under similar conditions.

(n) Represents the number of animals having detectable amount of PGDH activity from each group of six animals.

CONCLUSIONS

1. With low dose of diesel exhaust, a remarkable increase in PGDH activity was noted in guinea pigs only during the short exposure of 1.5 months.
2. With high dose, on the other hand, a clear time-dependent inhibition of PGDH activity was observed in guinea pig.
3. Further studies, varying the dose inversely in relation to the period of exposure, may reveal the threshold dose necessary for stimulation or inhibition of pulmonary PGDH activity of guinea pig.
4. The rat is not an ideal species for determination of effect of diesel exhaust on the PGDH activity since rat shows species difference in that it has about 10% of the enzyme activity compared to guinea pig.

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EFFECT OF DIESEL PARTICULATE EXPOSURE ON ADENYLATE AND GUANYLATE CYCLASE OF RAT AND GUINEA PIG LIVER AND LUNG

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ABSTRACT

Rats and guinea pigs were exposed to 250 or 1500 ug/m^3 diesel particulate for 24 weeks at the General Motors Research Laboratories. To determine the possibility of a tumor response from this exposure, we measured adenylate and guanylate cyclase activity of the liver and lung in these and control tissues. Although we found substantial qualitative differences throughout the 24 week period examined, we detected no changes in the basal activity of either enzyme system. Adenylate cyclase activity remains stimulatible from the liver and lung tissues of each species. Guanylate cyclase activity of the liver also remained stimulatible. On these findings, a tissue tumor response to these exposures was not supported. Age related changes were observed in the lung tissues of each species, and guanylate cyclase activity was decreased in this tissue after diesel exposure.

INTRODUCTION

The cyclic nucleotides are ubiquitous intracellular compounds found in most mammalian tissues as well as extracellular fluids (1, 4, 8, 9-10, 12). These compounds are the products of adenylate cyclase or guanylate cyclase and are susceptible to alteration by numerous hormones, neurotransmitter substances and drugs. In this study, we have examined the changes in these two intracellular enzymes in the liver and lung tissues of rats or guinea pigs subjected to a chronic exposure of diesel exhaust and particulate. This study was suggested by previous findings of others which demonstrated that these special enzymes and their cyclic nucleotide products are not only measures of the biologic viability of a tissue, but may also serve as an index in determining if a tissue contains a tumorous mass (5, 6, 11).

As it is now understood, under conditions of abnormal tissue growth these enzymes have been shown to be consistently changed when compared to normal tissue controls (5, 6, 11). While the cyclic nucleotides have been thought to act in concert, the changes which have been described from several studies consist of an increased basal activity, a substantial change of the guanylate cyclase enzyme from a soluble to a particulate pool, and the insensitivity of guanylate cyclase to chemical stimulation. In recent studies, the urinary excretion of cyclic nucleotides, and especially cGMP, was also found to be altered in benign or neoplastic tumors. Under these same conditions, cyclic AMP and adenylate cyclase activity was unchanged. Studies with in vivo tumors have suggested that the changes which are observed in cGMP metabolism may be associated with an altered rate of cellular growth and differentiation (5). These latter studies together with other studies involving in vivo transplantable tumors, suggest that the excretion of cyclic nucleotides in urine are consistent with changes in the cyclic nucleotide cyclase activities which are found in tumorous tissue. Such changes may not be a completely reliable index of tumors however. Changes in the responsiveness and in the basal activity of tissue cyclic nucleotide cyclases, can serve as sensitive indicators of the primary effects of a variety of noxious stimuli including smoke or the exhaust fumes from diesel engines.

Our studies have determined the activity of adenylate and guanylate cyclase in guinea pig or rat liver and lung tissues. Control animals as well as animals exposed to a low (250 ug/m^3), or high (1500 ug/m^3) concentrations of diesel particulate were examined. This paper reports the results of these diesel exposure after six weeks, twelve weeks or 24 weeks under conditions described by Schreck, et. al., and carried out at the General Motors Research Laboratories.

METHODS

Animal sacrifice and tissue preparation.

Animals (rats or guinea pigs) were first weighed, then stunned and decapitated. The liver and both lungs were removed and washed in a small beaker with approximately 50 ml cold saline. All tissues were blotted before weighing.

Liver microsomes were prepared by first passing the liver tissue through a Harvard press, followed by homogenization in four volumes of ice cold 0.25 M sucrose. Each homogenate volume was then measured and recorded. Ten ml of the homogenate was centrifuged for ten minutes at 12,000 rpm SS-34 rotor of a Sorvall centrifuge and the supernate carefully decanted. The supernate was then recentrifuged for 60 min at 40,000 rpm in a No. 40 Beckman rotor. Following this centrifugation, the resulting microsomal pellet was resuspended by hand using a teflon glass homogenizer in 16 ml of 0.25 M sucrose.

To prepare lung microsomes, each lung was filled with about 10 ml of cold saline and then cut into pieces with scissors. The fragments were homogenized in a Potter-Elvejham homogenizer using four volumes of 0.25 M sucrose. Homogenates were centrifuged for ten minutes at 12,000 rpm in an SS-34 rotor of a Sorvall centrifuge and the supernate decanted. The 12,000 rpm supernate was removed and recentrifuged at 40,000 rpm in a No. 40 Beckman rotor for an additional 60 minutes. The resulting microsomal pellet was resuspended by hand homogenization in one ml of 0.25 M sucrose.

Adenylate and guanylate cyclase protocol.

The reagent for adenylate cyclase was prepared according to the method of Steiner et al. (3). The reagent included, in final concentrations: 10 mM Tris-hydrochloride, pH 7.4, 3 mM ATP (adenosine triphosphate), 4 mM magnesium sulfate, 0.1 mM EGTA [ethyleneglycol-bis-(beta-aminoethyl ether)N,N′-tetra-acetic acid], 0.01 mM GTP (guanosine triphosphate) 2 mM DTT (dithiothreitol) and 3 mM IBMX (iso-butyl methyl xanthine). Maximal stimulation of adenylate cyclase was achieved by incubating purified liver or lung membranes in the adenylate cyclase reagent together with 1 mM (final concentration) sodium fluoride.

Guanylate cyclase reagent consisted of the following ingredients in final concentration: 50 mM Tris-hydrochloride, pH 7.6, 10 mM theophylline, 0.1 mM EDTA (ethylene diamine tetracetic acid), 0.1 mM DTT, 3.0 mM GTP, and 4.0 mM manganese chloride. Sodium azide (1 mM, final conc.) was used to maximally stimulate guanylate cyclase.

Adenylate cyclase activity was determined over a ten min period at 37° C. for both liver and lung using the 100,000 x g microsomal membrane pellet. Guanylate cyclase activity was determined over 10 min at 37° C. in liver and lung using both the 100,000 x g supernate and the microsomal pellet fractions. Assays for each time point for each of the tissues were determined in duplicate. To each assay tube, containing 100 ul of the appropriate reagent, 20 ul of tissue sample was added. All reagents and samples were held at 4° C. until incubations were started. To initiate the adenylate cyclase or guanylate cyclase activity, racks of tubes were placed into 37° C waterbaths. At 3, 10, or 15 min intervals, individual sample tubes were removed and quickly placed into a boiling waterbath (>95° C) for three min. Following this heat inactivation, 500 ul of 50 mM sodium acetate, pH 6.1, was added to all samples. Tubes were then capped and stored at -20° C. until assayed.

Radioimmunoassay of cyclic AMP and cyclic GMP.

Assay of cyclic nucleotides was performed using the double antibody radioimmunoassay (RIA) method described by Steiner, et. al. (7). Normalization of the cyclic nucleotide data was accomplished by protein determinations from individual samples to be assayed (15). Data reduction of the results of the assay were performed using a RIANAL package described by Duddleson, et. al. (13), kindly provided to this laboratory by Dr. A. Rees Midgley, University of Michigan, Division of Reproductive Endocrinology.

Data Reduction.

Correlation of the data obtained from the various experiments performed was determined using the Student T-test, the Studentized Neuman-Keuls test, and multivariate analysis of variance tests from packaged computer programs (14).

The specific activity of the control or treatment tissues was calculated from the averaged assayed activity of individual tissues. These averaged values were separately calculated by subtracting the 10 minute measurement from the zero minute incubation. Basal rates, as well as maximally stimulated enzyme activities were compared.

RESULTS

Six-Week Diesel Particulate Exposure.

Adenylate Cyclase:

Rats. The composite data of adenylate cyclase activities following 6-week exposure to diesel exhaust is shown in Table 1. A significant decrease is seen in the basal activity of the liver in those animals exposed to 250 ug/m^3 particulate. Stimulated activity of either the low or high exposure of diesel particulate was unchanged from the control values at this time. For the rat lung, a substantial decrease is noted in the basal activity of both the 250 and the 1500 ug/m^3 diesel particulate exposure. In contrast to the liver however, in the lung we observed an increase in the NaF-stimulatible adenylate cyclase, Table 1. The stimulatible adenylate cyclase activity observed following exposure to 250 ug/m^3 diesel particulate although increased, is unchanged from the control; after 6 weeks exposure to 1500 ug/m^3 however, a significant change is observed from control animals ($p < 0.01$). In addition, the increase observed following this highest exposure is significantly greater than that observed following 250 ug/m^3 exposure ($p < 0.03$).

Guinea Pigs. After 6 weeks exposure to diesel particulate, the basal levels of adenylate cyclase are nearly doubled (250 ug/m^3, $p < 0.05$, or more than tripled (1500 ug/m^3), $p < 0.01$, in the liver, Table 2. After either exposure to diesel particulate, NaF-stimulated adenylate cyclase is significantly greater, $p < 0.01$, than in the control tissues. In the guinea pig lung, a significant increase, $p < 0.01$, of more than six-fold is observed in the basal activity of adenylate cyclase after 250 ug/m^3 exposure. The activity of the enzyme remains significantly elevated, $p < 0.01$), and unchanged in animals receiving 1500 ug/m^3 exposure. In contrast to the liver, in the guinea pig lung, we find no differences in the NaF-stimulated activity compared to the control group of animals.

TABLE 1

EFFECT OF 6 WEEK DIESEL EXPOSURE ON ADENYLATE AND GUANYLATE CYCLASE

Animals	Condition	Adenylate Cyclase			Guanylate Cyclase	
		Liver	Lung		Liver	Lung
RATS	Control	17.8 ± 1.8	28.5 ± 4.6	S	6.0 ± 2.3	100.0 ± 24.1
		135.5 ± 18.1	205.6 ± 61.4		77.4 ± 10.9	110.5 ± 18.7
				P	0.20 ± 0.13	188.8 ± 22.6
					5.4 ± 0.9	182.8 ± 14.2
	Low Dose	5.5 ± 1.7	11.7 ± 3.7	S	33.9 ± 6.3	324.8 ± 38.0
		120.2 ± 10.1	266.4 ± 46.1		203.6 ± 18.2	297.7 ± 21.3
				P	N.D.	104.4 ± 7.1
					43.4 ± 2.8	134.8 ± 7.6
	High Dose	16.9 ± 3.6	12.3 ± 2.3	S	13.1 ± 1.6	97.2 ± 12.8
		190.0 ± 53.1	366.0 ± 36.2		71.2 ± 3.8	96.8 ± 17.0
				P	N.D.	65.5 ± 4.8
					29.9 ± 1.4	59.5 ± 3.6

* Specific Activity (picomoles/mg protein/min) ± S.E.M., based on 10 minute cyclase determinations. Adenylate cyclase activity stimulated by NaF, guanylate cyclase activity stimulated by NaN(3). Data in this and other tables expressed as: basal activity (unstimulated/stimulated enzyme activity.

TABLE 2

EFFECT OF 6 WEEK DIESEL EXPOSURE ON ADENYLATE AND GUANYLATE CYCLASE

Animals	Condition	Adenylate Cyclase		Guanylate Cyclase		
		Liver	Lung		Liver	Lung
GUINEA PIG	Control	5.4 ± 1.1	3.3 ± 2.4	S	5.0 ± 0.3	29.0 ± 3.2
		87.3 ± 4.1	106.2 ± 13.5		82.1 ± 3.3	31.8 ± 1.6
				P	0.8 ± 0.3	28.2 ± 3.8
					5.0 ± 1.8	30.4 ± 4.2
	Low Dose	9.1 ± 1.9	21.0 ± 4.9	S	5.7 ± 0.8	53.2 ± 4.5
		132.2 ± 19.9	106.8 ± 12.9		115.2 ± 7.6	81.8 ± 4.9
				P	N.D.	67.6 ± 4.6
					20.1 ± 3.2	74.9 ± 6.2
	High Dose	18.5 ± 3.5	15.3 ± 1.7	S	6.7 ± 1.1	61.5 ± 5.9
		154.8 ± 27.0	89.6 ± 13.4		172.2 ± 8.5	50.9 ± 4.1
				P	N.D.	64.2 ± 5.1
					23.3 ± 4.3	63.1 ± 9.8

* Specific Activity (picomoles/mg protein/min) ± S.E.M., based on 10 minute cyclase determinations. Adenylate cyclase activity stimulated by NaF, guanylate cyclase activity stimulated by NaN(3).

Guanylate Cyclase:

Rat. The basal activity of the rat liver 100,000 x g supernate fraction was increased more than five-fold, $p < 0.01$, following 6-weeks exposure to 250 ug/m^3 diesel particulates. This same fraction was more than doubled in those animals which received 1500 ug/m^3 exposure, Table 3. When liver soluble guanylate cyclase was maximally stimulated with sodium azide, a significant increase was only observed in those animals exposed to 250 ug/m^3 of diesel particuate. While no differences could be detected in the basal activity of the corresponding particulate fractions, significant differences, $p < 0.01$, were determined for stimulated guanylate cyclase activity after either 250 or 1500 ug/m^3 diesel exposures. In the rat lung, basal levels of soluble guanylate cyclase are unchanged from enzyme activity which was maximally stimulated by sodium azide. The approximately 3-fold increase in basal and stimulated levels of soluble guanylate cyclase activity from animals exposed to 250 ug/m^3 diesel particulate, matches the general increase of guanylate cyclase activity seen in liver of these same animals. When the particulate guanylate cyclase of the rat lung is examined, a progressive decrease in enzyme activity is noted, with increasing diesel exposure concentrations. The particulate guanylate cyclase activity show stimulated enzyme activities which are essentially unchanged from the basal rates.

Guinea Pig. While no change is observed in the basal activity of the guinea pig liver after 6 weeks of diesel exposure at either concentration, a dose related increase in the sodium azide stimulatible guanylate cyclase activity is observed after diesel exposure, Table 4. Unstimulated particulate guanylate cyclase activity of guinea pig liver is unchanged after any exposure to diesel particulate; however the maximal stimulated particulate activity is significantly increased, $p < 0.01$, by approximately 4 fold after either 250 or 1500 ug/m^3 diesel exposure, Table 4. Basal activity of soluble guanylate cyclase activity of guinea pig lung is increased, approximately 2-fold, $p < 0.02$, after either concentration of diesel exposure. The maximal stimulated soluble guanylate cyclase activity is nearly doubled at this time. A similar two-fold increase is also observed in the basal and stimulated activity of the particulate guanylate cyclase in the lung, Table 5.

Twelve-Week Diesel Particulate Exposure.

TABLE 3

EFFECT OF 12 WEEK DIESEL EXPOSURE ON ADENYLATE AND GUANYLATE CYCLASE

Animals	Condition	Adenylate Cyclase		Guanylate Cyclase		
		Liver	Lung		Liver	Lung
RAT	Control	5.6 ± 2.8	9.4 ± 2.1	S	1.4 ± 0.5	12.6 ± 3.3
		57.6 ± 3.6	127.2 ± 6.5		28.4 ± 6.7	10.5 ± 2.2
				P	2.5 ± 0.8	7.7 ± 4.6
					4.6 ± 1.4	14.6 ± 2.3
	Low Dose	3.3 ± 1.0	9.5 ± 2.3	S	4.4 ± 1.0	6.6 ± 0.9
		106.1 ± 12.8	90.6 ± 14.7		21.4 ± 3.1	12.5 ± 1.8
				P	5.6 ± 1.3	12.2 ± 2.0
					29.0 ± 1.9	12.1 ± 2.5
	High Dose	13.5 ± 1.9	21.4 ± 1.5	S	18.3 ± 2.9	51.1 ± 5.1
		167.6 ± 16.0	101.1 ± 22.8		30.2 ± 1.9	61.3 ± 6.6
				P	5.2 ± 1.3	47.8 ± 2.1
					9.5 ± 2.4	51.3 ± 2.2

* Specific Activity (picomoles/mg protein/min) ± S.E.M., based on 10 minute cyclase determinations. Adenylate cyclase activity stimulated by NaF, guanylate cyclase activity stimulated by NaN(3).

TABLE 4

EFFECT OF 12 WEEK DIESEL EXPOSURE ON ADENYLATE AND GUANYLATE CYCLASE

Animals	Condition	Adenylate Cyclase		Guanylate Cyclase	
		Liver	Lung	Liver	Lung
GUINEA PIG	Control	25.5 ± 1.4 203.3 ± 31.4	8.0 ± 3.2 93.2 ± 14.7	S 16.9 ± 1.7 131.0 ± 6.1 P 4.3 ± 1.2 22.5 ± 2.9	50.4 ± 3.3 60.2 ± 2.1 67.0 ± 1.5 63.7 ± 3.7
	Low Dose	15.7 ± 7.6 288.0 ± 30.1	4.2 ± 2.1 85.0 ± 12.8	S 7.2 ± 0.6 157.7 ± 5.9 P 16.2 ± 2.5 79.9 ± 8.4	64.0 ± 9.3 63.4 ± 8.2 43.8 ± 2.9 53.0 ± 5.8
	High Dose	6.9 ± 1.6 89.6 ± 11.4	3.9 ± 0.9 60.2 ± 13.9	S 19.1 ± 3.7 153.0 ± 4.4 P 20.2 ± 4.4 29.8 ± 2.3	48.0 ± 4.2 48.4 ± 4.9 54.5 ± 1.6 56.0 ± 2.6

* Specific Activity (picomoles/mg protein/min) ± S.E.M., based on 10 minute cyclase determinations. Adenylate cyclase activity stimulated by NaF, guanylate cyclase activity stimulated by NaN(3).

TABLE 5

EFFECT OF 24 WEEK DIESEL EXPOSURE ON ADENYLATE AND GUANYLATE CYCLASE

Animal	Conditions	Adenylate Cyclase			Guanylate Cyclase	
		Liver	Lung		Liver	Lung
RAT	CONTROL	8.7 ± 0.6	52.2 ± 5.4	S	120 ± 1.8	73.4 ± 6.2
		44.2 ± 4.3	226.0 ± 45.5		41.1 ± 4.5	75.8 ± 14.7
				P	N.D.	119.7 ± 37.6
					15.9 ± 2.4	86.2 ± 28.1
	Low Dose	4.9 ± 0.9	34.6 ± 1.8	S	7.8 ± 2.0	1.7 ± 0.4
		42.9 ± 2.6	219.7 ± 15.6		38.5 ± 5.3	15.4 ± 3.1
				P	6.3 ± 1.0	35.8 ± 4.9
					530.0 ± 25.3	45.7 ± 3.5
	High Dose	6.5 ± 1.3	17.5 ± 2.3	S	8.8 ± 3.0	74.5 ± 10.0
		28.0 ± 2.8	971.6 ± 5.1		197.5 ± 28.3	368.6 ± 38.8
				P	4.5 ± 0.4	47.4 ± 8.6
					235.1 ± 34.2	142.6 ± 39.3

* Specific Activity (picomoles/mg protein/min) ± S.E.M., based on 10 minute cyclase determinations. Adenylate cyclase activity stimulated by NaF, guanylate cyclase activity stimulated by NaN(3).

Adenylate Cyclase:

Rat. In the rat liver, adenylate cyclase is unchanged following a 12-week exposure at 250 ug/m^3, but is increased more than two-fold in those animals exposed to 1500 ug/m^3, Table 3. In the same tissue the maximum sodium fluoride stimulatible activity is doubled (250 ug/m^3) or tripled (1500 ug/m^3) after diesel exposure. Basal adenylate cyclase activity in the rat lung shows a similar pattern of response, and is increased more than two-fold in those animals exposed to 1500 ug/m^3 diesel particulate. In contrast to liver, however, the lung NaF-stimulatible adenylate cyclase activity remains unchanged following diesel particulate exposure.

Guinea Pigs. Adenylate cyclase activity in the liver of the guinea pig is progressively decreased by increasing exposure to diesel particulate, Table 4. The sodium fluoride stimulatible adenylate cyclase activity is significantly increased above controls in the liver membranes of animals which have been exposed to 250 ug/m^3 of diesel particulate. Animals exposed to 1500 ug/m^3 diesel particulate showed a dramatic decrease in sodium fluoride stimulatible adenylate cyclase activity from both the controls or animals exposed to 250 ug/m^3 of diesel particulate. In the lung of the guinea pig, exposure to either concentration of diesel particulate reduced the basal level of the adenylate cyclase activity by one-half. Maximal adenylate cyclase activity following sodium fluoride stimulation was unchanged from the control groups after either diesel exposure.

Guanylate Cyclase:

Rat. The basal activity of soluble guanylate cyclase for the rat liver is increased three-fold (250 ug/m^3) or more than ten-fold (1500 ug/m^3), Table 3. At the same time, the maximal stimulation of soluble guanylate cyclase activity remains unchanged from control levels after exposure to diesel particulate. Guanylate cyclase activity associated with the particulate fraction from the rat liver is approximately doubled following exposure to either diesel exposure. The maximum sodium azide stimulated guanylate cyclase activity was increased by more than five-fold (250 ug/m^3) and by two-fold (1500 ug/m^3) at this time. In the rat lung, the basal activity of soluble guanylate cyclase activity was increased more than four-fold in those animals exposed to 1500 ug/m3 diesel particulate.

Only stimulatible guanylate cyclase activity was observed in those animals exposed to 250 ug/m^3 diesel particulate. Guanylate cyclase activity associated with the lung membrane fraction was found to be increased six-fold in the animals exposed to 1500 ug/m^3 diesel particulate.

Guinea Pig. The basal activity of soluble guanylate cyclase activity in the guinea pig liver, although variable after exposure to low concentrations of diesel particulate, remained unchanged from control, Table 4. Maximum soluble guanylate cyclase activity was increased significantly after either concentration of diesel particulate exposure. Basal activity of particulate guanylate cyclase from the guinea pig liver demonstrated approximately four-fold increase in activity after exposure to either concentration of diesel particulate. The maximal stimulated soluble or particulate guanylate cyclase activities were identical to response patterns in the rat: after 250ug/m^3, a more than three-fold increase of stimulated activity is observed, while exposure at 1500 ug/m^3 remains essentially unchanged from control values. In the guinea pig lung, basal activity of the soluble guanylate cyclase activity is unchanged from control after any exposure to diesel particulate. In the same tissues, there is no increase in guanylate cyclase activity on exposure to sodium azide which should maximally stimulate the enzyme in the soluble fraction. The basal particulate guanylate cyclase activity is decreased after exposure to either concentration of diesel particulate. This particulate enzyme follows the same pattern of non-stimulation when identical tissue extracts are treated with sodium azide.

Twenty-four Week Diesel Particulate Exposure.

Adenylate Cyclase:

Rats. After twenty-four weeks of diesel particulate exposure, the basal adenylate cyclase activity from rat liver membranes, although slightly decreased, was unchanged from the control values, Table 5. At the same time, while no differences could be detected in maximal sodium fluoride stimulatible adenylate cyclase activity after a low exposure to diesel particulate, a significant decrease was observed, $p < 0.05$, in those animals which received 1500 ug/m^3 diesel exposure. In the rat lung, the basal activity of adenylate cyclase was significantly decreased at both 250 (66% of control), $p < 0.05$, and at 1500 (33% of control), $p <$

0.01, diesel exposure concentrations. In this tissue, like the liver, while maximal sodium fluoride stimulated guanylate cyclase activity remained equal to the control in the animals exposed to 250 ug/m^3 diesel particulate, after 1500 ug/m^3 it was significantly reduced.

Guinea Pig. Studies of adenylate cyclase activity from guinea pig liver, show differences only in those animals exposed to 1500 ug/m^3 of diesel particulate, Table 6. In this treatment, both the basal activity, $p < 0.05$, as well as the stimulated activity, $p < 0.02$, is significantly increased from control values. In the guinea pig lung, basal adenylate cyclase activity is significantly decreased after any exposure to diesel particulate, $p < 0.05$. Maximally stimulated adenylate cyclase activity of the lung is somewhat decreased after 250 ug/m^3 exposure, and is significantly decreased after 1500 ug/m^3 exposure.

Guanylate Cyclase:

Rat. Basal activity of the soluble guanylate cyclase from the liver is significantly decreased, $p < 0.05$, after 250 ug/m^3 diesel exposure, Table 5. The comparable maximally stimulated activity of this soluble fraction is unchanged (250 ug/m^3) or increased more than four-fold (1500 ug/m^3) at this time. Comparison of basal particulate guanylate cyclase activity is unremarkable in the liver. Particulate guanylate cyclase activity in the rat liver which is stimulated by sodium azide is increased 33-fold by exposure to 250 ug/m^3 diesel exposure, and by more than fourteen-fold after 1500 ug/m^3 diesel exposure, Table 6. In the lung, soluble guanylate cyclase activity is unchanged from control after 250 ug/m^3 exposure. However, while the basal soluble guanylate cyclase activity is unchanged from the control levels after exposure to 1500 ug/m^3 of diesel particulate, a sodium azide stimulated activity of nearly five-fold increase is now observed. A similar pattern is seen for the particulate guanylate cyclase activity in this tissue.

Guinea Pig. In the liver, while no changes from control is observed in basal guanylate cyclase activity after 250 ug/m^3 diesel exposure, a substantial decrease is observed after 1500 ug/m^3 exposure, Table 6. Maximally stimulated soluble guanylate cyclase activity, shows a tendency to decrease, but remains essentially unchanged from control after either diesel exposure. When particulate guanylate cyclase activity

TABLE 6

EFFECT OF 24 WEEK DIESEL EXPOSURE ON ADENYLATE AND GUANYLATE CYCLASE

Animal	Conditions	Adenylate Cyclase			Guanylate Cyclase	
		Liver	Lung		Liver	Lung
GUINEA PIG	Control	4.5 ± 1.6	19.7 ± 2.2	S	11.2 ± 1.2	114.3 ± 9.8
		28.8 ± 2.3	160.3 ± 7.9		292.6 ± 48.7	128.1 ± 14.3
				P	7.5 ± 2.3	62.0 ± 9.8
					505.8 ± 128.0	174.4 ± 28.1
	Low Dose	4.5 ± 1.3	14.0 ± 1.4	S	14.9 ± 1.7	71.5 ± 12.0
		33.6 ± 3.5	139.2 ± 11.0		238.4 ± 5.8	112.8 ± 19.8
				P	18.5 ± 4.3	75.2 ± 2.7
					53.3 ± 4.8	116.0 ± 7.5
	High Dose	9.4 ± 1.1	12.5 ± 1.8	S	3.5 ± 1.6	5.3 ± 1.0
		47.1 ± 2.4	102.5 ± 8.3		205.9 ± 23.1	44.3 ± 7.6
				P	75.5 ± 21.6	79.5 ± 7.5
					100.0 ± 31.3	107.4 ± 11.7

* Specific Activity (picomoles/mg protein/min) ± S.E.M., based on 10 minute cyclase determinations. Adenylate cyclase activity stimulated by NaF, guanylate cyclase activity stimulated by NaN(3).

is examined, a doubling (250 ug/m^3) or an increase of nearly ten-fold (1500 ug/m^3) is observed. At this same time, the marked activity of the particulate guanylate cyclase in the guinea pig liver is reduced nearly ten-fold (250 ug/m^3) or one-fifth (1500 ug/m^3). In the guinea pig lung, an extremely active basal soluble guanylate cyclase activity is reduced 38% by 250 ug/m^3 diesel exposure, and by 95% after 1500 ug/m^3 diesel exposures. The maximally stimulated activity in this tissue after 1500 ug/m^3 diesel exposure is reduced to one-third of the control activity. When particulate guanylate cyclase activity of the guinea pig lung is compared, we find no changes in basal activity compared to control animals. However, maximally stimulated particulate guanylate cyclase activity of the guinea pig lung is significantly reduced, $p < 0.02$ or greater, by exposure to either concentration of diesel particulate.

DISCUSSION

From the studies performed to date, there are indications of changes which have occurred not only in the basal activity of adenylate and guanylate cyclase, but also in the responsiveness of these enzymes to maximal stimulation after diesel particulate exposure. The most obvious changes are those in which the basal activity of the tissues is increased with exposure to diesel exhaust when compared to control animals and tissues. This sequence was associated primarily with the studies of guanylate cyclase activity, and occurred in both the rat and the guinea pig species. Although present after 6 weeks of diesel exposure, the trend was more pronounced in the data observed after 3 months of exposure. Another trend is the decrease which we find in basal (unstimulated) adenylate cyclase activity in each of the species following increasing diesel particulate exposure. Because of large deviations in these data groups however, this information is not as apparent as the data related to guanylate cyclase.

The above patterns are evident throughout the first 24 weeks of diesel exposure to the rat and guinea pigs. These patterns are also distinctive for both the animal species and the tissue examined.

When compared to control animals, rat liver adenylate cyclase activity is unchanged after either 6 weeks or 24 weeks of exposure. At 12 weeks, there is a marked exposure related increase in NaF-stimulatible adenylate cyclase. In the same tissue, there is a marked reduction in stimulatible adenylate cyclase after 24 weeks in all animals tested. In the lung, an initial dose-related increase in NaF-stimulatible adenylate cyclase activity is abated so that there are no changes observed after 12 weeks of exposure. After 24 weeks of diesel exposure, a significant reduction in stimulatible adenylate cyclase activity is observed in lung tissue.

Liver adenylate cyclase activity in the guinea pig, which shows a marked increase in NaF-stimulatible activity after 6 weeks of diesel exposure, is further increased at 12 weeks but is substantially reduced after 24 weeks of exposure. In the lung, there is a definite trend to increase the basal rate of adenylate cyclase activity with age. More importantly, a clear trend is evident which demonstrates a reduction in NaF-stimulatible adenylate cyclase activity with diesel exposure. In the soluble guanylate cyclase activity of the rat lung, beginning after 12 weeks of exposure and increasing following 24 weeks of exposure, we observed the appearance of guanylate cyclase which is readily stimulated by sodium azide. The particulate guanylate cyclase activity of rat liver shows a stimulatible pool of enzyme present after both 6 and 12 weeks of diesel exposure; after 24 weeks, a dramatic change in stimulatible guanylate cyclase activity is observed. The particulate guanylate cyclase activity of the lung provides a clear trend throughout all treatments of a decrease in basal activity. In addition, a significant trend of increased stimulatible guanylate cyclase is observed with increased diesel exposure and time.

Soluble guanylate cyclase activity in the guinea pig liver shows an initial trend of increased activity following diesel exposure, through week 12 of the exposure study. After 24 weeks however, this initial stimulation is reversed in an apparently dose related pattern. Soluble guanylate cyclase activities in the guinea pig lung suggest a progressive loss in basal activity, and the appearance of a stimulatible fraction with age. This stimulatible fraction in unaffected by diesel exposure. The findings which we observe in guinea pig guanylate cyclase liver particulate guanylate cyclase activity demonstrates a marked trend toward increased basal activity seen most clearly after 24 weeks exposure. Basal particulate guanylate cyclase activity in the guinea pig lung remains unchanged throughout 24 weeks of exposure. Interestingly, after 24 weeks of exposure, a stimulatible guanylate cyclase activity appears in guinea pigs. This age-related stimulatible activity is significantly reduced by exposure to diesel particulate.

Conclusions: In the findings reported to date, we find no evidence of the trend of increased basal activity of adenylate cyclase or guanylate cyclase following exposure to diesel exhaust particulate. Throughout the 24 weeks of exposure reported, although substantial qualitative differences are apparent, adenylate cyclase activity remains stimulatible from the rat or guinea pig liver and lung tissues. Guanylate cyclase activity also retains stimulation effects in both the rat and guinea pig liver. On the basis of these studies therefore, a conclusion of tumorous response to diesel exposure cannot be supported. In other observations, in the lung of each species, we find an age-related appearance of stimulatible activity. This stimulatible activity is depressed in the presence of diesel exposure. And finally, the data of this study supports the conclusion that some loss of stimulatible guanylate cyclase activity in the lung, while showing a stimulation of this enzyme in the liver of the rat and guinea pig.

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BIOCHEMICAL ALTERATIONS IN LUNG CONNECTIVE TISSUE IN RATS AND MICE EXPOSED TO DIESEL EMISSIONS

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ABSTRACT

With increasing use of diesel-powered vehicles, it is necessary to assess the potential hazards of increased diesel emissions in the environment.

The lung is the primary portal of entry of atmospheric contaminants in the body. Lung structure and function are dependent on the structural protein collagen, and chemical injury to the lung elicits a connective tissue response involving alterations in collagen content and synthesis.

We have examined the effect of inhaling diesel emissions on lung collagen synthesis in rats and mice. our studies showed that exposure to diesel emissions increased the rate

of collagen synthesis and the levels of prolyl hydroxylase, an enzyme intimately associated with collagen synthesis. These observations suggest that diesel emissions may induce connective tissue alterations in lungs.

INTRODUCTION

The increasing popularity and use of diesel-powered vehicles makes it essential to assess the possible hazards of diesel emissions. The lungs are likely to be a primary target for any toxic effects of such emissions since they are not only the primary portal of entry of airborne contamnants in the body, but they also present the largest exposed surface.

The structure and function of lungs are dependent on connective tissue which comprises a large part of lung mass. Collagen, the principal structural protein of connective tissue, is responsible for maintaining the architectural integrity of the lungs, and it actively participates not only in the mechanical aspects of lung function, but is also a part of the blood-gas interphase (1). Aberration in collagen content, distribution, and synthesis accompany pulmonary dysfunction induced by a variety of atmospheric toxins (1,2).

We have examined the synthesis of collagen and overall protein synthesis in lungs of mice and rats exposed to diesel emissions. In our studies, inhalation of diesel emissions significantly altered these parameters of lung macromolecular synthesis on experimental animals, suggesting that diesel emissions may induce structural changes in lungs, and contribute to pulmonary dysfunction.

MATERIALS AND METHODS

Experimental Animals

Male Sprague-Dawley rats (CD Strain), 10 weeks of age, were obtained from Charles River Breeding Laboratories. Strain A/HEJ mice were obtained from the same source. The animals were maintained on a rodent diet (Purina) and with free access to water.

Radiochemicals

L-[3,4-^{3}H]-proline, 25 Ci/mmol, L-[^{14}C(U)]-proline, 285 mCi/mmol and L-[4,5-^{3}H]-leucine, 100 Ci/mmol were products of New England Nuclear.

Exposure to Diesel Emissions

Exposure to diesel emissions was carried out as described in detail elsewhere (3,4).

Biochemical Procedures

In vivo labelling of proteins with radioactive leucine or proline was carried out in rats by injecting the radioactive precursor in the tail vein, at a concentration of 1 mCi/Kg weight of the animal.

In vitro labelling of tissues was carried out by incubating the minced tissues in short-term organ culture in Dulbecco-Vogt medium in a shaker-water bath maintained at 37°. The tissue-mince was suspended in the medium in a ratio of 5 ml medium/g tissue. The medium contained 10 Ci of radioactive proline. Incubation was carried out for 3 hours and terminated by rapidly chilling and homogenizing the tissues in a Polytron.

Homogenates of tissues labelled *in vivo* or *in vitro* were extensively dialyzed to remove free radioactivity and hydrolyzed in 6N HCl for assay for collagen and total protein. Collagen synthesis was assayed by analyzing the hydrolyzate for labelled hydroxyproline (5), and total protein content was assayed by determining the total ninhydrin reactive material, using leucine standards (6). Radioactive incorporation data were expressed as specific activity terms based on dpm incorporated/ Mol leucine equivalent.

Prolyl Hydroxylase Assay

Lungs from animals maintained in clean air or diesel-contaminated environments were homogenized in a Polytron in 0.1 M TRIS-HCl, pH 7.4, containing 0.1 M KCl and 0.1% Triton-X-100. Aliquots of the 15,000 G supernatents of the homogenates were assayed for prolyl hydroxylase as described elsewhere (7). The protein content of the supernatent was assayed by the Folin-Lowry procedure. The assay for prolyl hydroxylase is based on the release of ^{3}H from ^{3}H-proline-labelled unhydroxylated collagen. Enzyme activity was, therefore, expressed in terms of dpm^{3}H released/mg protein in the supernatent.

RESULTS AND DISCUSSION

Physical Appearance and Characteristics of Exposed Lungs

When the animals were dissected, it became apparent that the lungs of animals maintained in diesel-contaminated environments were markedly different both in appearance and in physical characteristics such as elasticity and consistency. In general, the exposed lungs were considerably enlarged, no longer pink, and appeared grety to black as the length of exposure was increased. Many lungs appeared mottled and some had distinct nodules visible to the naked eye. The homogenates of exposed lungs were sooty and oily in appearance. These observations are summarized in Table I for exposed and unexposed mice. Similar differences were observed in case of rat lungs.

Protein Content and Protein Synthesis

The exposed lungs were enlarged both in terms of volume, measured crudely by displacement of water in a graduated cyliner, and also in terms of wet weight. The protein content of the exposed lungs was markedly elevated. As seen in Table II, the total protein content of rat lungs exposed to diesel emissions for 42 days was increased nearly 40%. Increased protein content in lung injury can result from increased accumulation of circulating proteins and migratory cells in the alveolar interstitium and in other compartments. Protein content may also increase as a result of connective tissue proliferation, and the increased deposition of connective tissue matrix proteins.

In order to determine if there was a change in the rate of overall protein synthesis in the lungs, the tissues were pulse-labelled *in vivo*, by intravenous injection of ^{3}H-leucine. Previous experiments had demonstrated that in the lung the highest specific activity of unincorporated radioactive precursors administered through the tail vein, was achieved between 15 and 30 minutes. In these experiments, the pulsed incorporation was terminated at 20 minutes after the radioactive precursor was administered, by sacrificing the animals and rapidly freezing the tissues.

In this experiment, tissues from anmals in each group were pooled before analysis. As seen in Table III, the specific activity of leucine incorporation was lower in the exposed animals than in controls. Even if it is assumed that there is some dilution of label by accumulated protein in the lung, this observation suggests a decrease in the overall synthesis of proteins. Decreased protein synthesis has been observed in other forms of chemical injury to the lungs (2).

The synthesis of collagen was examined under the same conditions by injecting ^{3}H-proline and following the incorporation of total ^{3}H-radioactivity and the synthesis of ^{3}H-hydroxyproline in the lungs. Data from this experiment are presented in Table IV. Unlike leucine, which is considerably less abundant in collagen than in other proteins, the incorporation of ^{3}H-proine and the synthesis of ^{3}H-hydroxyproline did not appear to be significantly altered on exposure to diesel emissions. Proline is several times more abundant in collagen than in noncollagen proteins, and significant amounts of hydroxyproline occur only in collagen. These data can thus be interpreted as suggesting a relative increase in collagen synthesis in comparison to the synthesis of noncollagen proteins in lungs exposed to diesel emissions.

Increased collagen synthetic activity in the exposed lungs was also confirmed in experiments in whcih lung tissues were labelled in short-term organ culture. In this experiment, lungs were dissected out and minced in a small volume of organ culture medium, and rinsed several times to eliminate the accumulated circulating protein material, before being labelled with ^{14}C-proline. As seen in Table V, although the overall incorporation of the radioactive precursor was suppressed, the ratio of radioactivity in hydroxyproline to the total radioactive incorporation was nearly doubled. These data are consistent with the in vivo incorporation studies and suggest that, although there is decreased protein synthesis in diesel-exposed rat lungs, the proportion of collagen synthesis is greater.

Prolyl hydroxylase is a crucial enzyme in the pathway of collagen synthesis, and its levels usually reflect a tissues potential for collagen synthesis. Prolyl hydroxylase levels in lungs are elevated when the tissue is injured and such increases reflect increased turnover and proliferation of connective tissue (8-10). Increased prolyl hydroxylase activity in exposed lungs would be consistent with injury. We examined prolyl hydroxylase activity in young adult male and female rats exposed to diesel emissions for 42 days, and in rat pups, exposed to diesel emissions either in utero or neo-nataly. These groups exhibited marked differences in their response to diesel emissions as measured by alterations in prolyl hydroxylase activities (Table VI). After 42-day exposure, adult female rats showed larger increases in lung prolyl hydroxylase than did the adult male rats; however, there was a greater increase in protein accumulation in the lungs of male rats, and this makes the data on male rat lungs difficult to interpret. 20 day old rats exposed to diesel emissions in utero showed significant increases in prolyl hydroxylase activity. These data suggest that toxic components of diesel emissions may pass the placental barrier. Interestingly, when neo-natal rats were exposed to diesel emissions for 20 days, there appeared to be a decrease in lung prolyl hydroxylase. The reason for this decrease is not clear at present. One possible explanation is that large accumulation of infiltrating proteins may have lowered the specific activity of the enzyme.

We also examined the effect of length of exposure to diesel emissions on lung collagen synthesis. These experiments were carried out using male mice of A/HEJ Strain. The animals were exposed to diesel exhaust for varying periods. The lungs exhibited marked changes in appearance and other characteristics as summarized in Table I. They were examined for collagen and protein synthesis in short-term organ cultures (Table VII). As in the case of rat lungs, there appeared to be large increases in protein content in the exposed tissues, and while the specific activity terms do not accurately reflect lung protein synthesis in these experiments, the rate of protein synthesis appeared to be lowered. The sysntehsis of collagen, however, appeared to account for a greater proportion of total protein synthesized, and increased with the length of exposure so that, after a 9-month exposure, the rate of collagen synthesis was 1.5 times that in the controls. These data support the observations made in the experiments with rats, and, furthermore, they suggest that continual exposure to diesel emissions may exacerbate lung injury. These studies also suggest a species difference in the susceptibility to the deleterioius effects of diesel emissions in that mice may be more resistant to such injury than rats.

Our *in vivo* studies on the effect of diesel emissions on lung collagen synthesis are corroborated by other studies in our laboratory using rat lung organ cultures (11-13). Some of these studies are summarized in another contribution from our laboratory at the symposium (13). Polyaromatic hydrocarbons are the major toxic components of diesel emissions (14). When lung organ cultures were exposed to benzo(a)-pyrene for periods of 24 hours, they exhibited markedly increased activities of prolyl hydroxylase (11), and synthesized collagen at elevated rates (12,13). Furthermore, the relative proportion of type I and type III collagen synthesis were altered (12,13).

Table I

PHYSICAL CHANGES IN LUNGS OF MICE EXPOSED TO DIESEL EMISSIONS

	Color	Approx. Size	Nodes	Insoluble White Sinewy Material After Homogenization
3.5 Month				
Control	Pink	100%	-	-
Exposed	Gray/Black Pink Edges	120%	+++	+
6.0 Month				
Control	Pink	100%	-	-
Exposed	Black with Some Pink Edges	150%	++++	++
9.0 Month				
Control	Gray/Pink	120%	+	-
Exposed	Black with White Edges	180%	+++	+++

Size = Wet Weights and Volume.

Table II

TOTAL PROTEIN CONTENT OF LUNGS

	Protein mg/g Tissue (Wet Wt.)	% Change
Control	54	-
42-Day Diesel Exposure	71	31
42-Day Diesel Exposure (12 Animals/Group)	78	44

The total protein content of the lungs was assayed on the basis of total ninhydrin reactive material in tissue hdrolyzates. In order to express the protein content in these units (mg/g tissue), the assay was based on bovine serum albumin used in standards adn hydrolyzed and processed identically to the tissues.

Table III

PULSE-LABELLED INCORPORATION OF ^{3}H-LEUCINE INTO LUNG PROTEIN

	10^{-5}xdpm ^{3}H/g Tissue (Wet Wt.)
Control	1.17
42-Day Diesel Exposure	0.72
42-Day Diesel Exposure (12 Animals/Group)	0.56

The incorporation of ^{3}H-leucine into lung proteins was assayed in the hydrolyzates and related to the protein content as described in the legend to Table I.

Table IV

DIESEL EXPOSED RATS - RADIOACTIVE PULSE LABELLED IN VIVO PROLINE INCORPORATION AND HYDROXYPROLINE SYNTHESIS

Code No.	$\frac{\text{Total HO-Pro DPM}}{\text{Total Protein DPM}}$	Specific Activity $\frac{\text{Total Protein DPM}}{\text{Mole Leucine Equiv}}$	Specific Activity $\frac{\text{Total HO-Pro DPM}}{\text{Mole Leucine Equiv}}$
19-Control	3.07 ± 0.51	244 ± 58	6.87 ± 1.11
10-Exposed	2.80 ± 0.58	222 ± 77	6.40 ± 2.64
6-Exposed	2.62 ± 0.47	197 ± 43	5.40 ± 2.36

Animals were intravenously (tail vein) injected with 1000 Ci proline [3,4-^{3}H(N)] per kilogram. The animals were sacrified 20 minutes after injection. Lungs perfused with cold saline, removed, weighed, and frozen. The samples were thawed, homogenized, dialyzed against running water, hydrolyzed, and analyzed for HO-Pro content by the Prockop method.

Table V

DIESEL EXPOSED RATS - RADIOACTIVE PULSE LABELLED IN VIVO PROLINE INCORPORATION AND HYDROXYPROLINE SYNTHESIS

Code No.	$\frac{\text{Total HO-Pro DPM}}{\text{Total Protein DPM}}$	Specific Activity $\frac{\text{Total Protein DPM}}{\text{Mole Leucine Equiv}}$	Specific Activity $\frac{\text{Total HO-Pro DPM}}{\text{Mole Leucine Equiv}}$
Control	4.85 ± 1.05	163 ± 45	6.66 ± 1.62
Exposed	8.30 ± 2.23	48 ± 23	3.21 ± 1.73

Tissues were labelled in vitro in short-term organ cultures as described, homogenized, dialyzed, and the 6N HCl hydrolyzates assayed for radioactive incorporation and the total ninhydrin content as a measure of protein content.

Table VI

PROLYL HYDROXYLASE LEVELS IN LUNGS OF RATS EXPOSED TO DIESEL EXHAUST

	Prolyl Hydroxylase Activity (DPM ^{3}H/mg Protein)
Female Control (6 Rats)	3730 ± 1086
Female Exposed (6 Rats)	5965 ± 1332 (p 0.05)
Male Control (6 Rats)	561 ± 1522
Male Exposed (6 Rats)	4780 ± 1744 (p 0.05)
Gestational Control (20 Rats)	5474 ± 1435
Gestational Exposed (20 Rats)	7342 ± 1534 (p 0.0005)
Neo-Natal Control (19 Animals)	6644 ± 1321
Neo-Natal Exposed (18 Animals)	3051 ± 376 (p 0.0001)

Lungs were homogenized in a Polytron homogenizer and pH activity was determined in 15,000 xg supernatant by measuring the release of ^{3}H from ^{3}H-Pro-labelled unhydroxylated collagen. Each number is the average of six determinations.

Table VII

DIESEL EXPOSED MICE LUNGS LABELLED IN VITRO PROLINE INCORPORATION AND HYDROXYPROLINE SYNTHESIS

	Total HO-Pro DPM x 10^2 / Total Protein DPM	Specific Activity Total Protein DPM / Mole Leucine Equiv	Specific Activity Total HO-Pro DPM / Mole Leucine Equiv
3.5 Month			
Control	1.58 ± 0.30	8919 ± 512	151 ± 45
Exposed	1.86 ± 0.41 (+19%)^{a}	4070 ± 320^{b}	89 ± 5^{b}
6.0 Month			
Control	3.24 ± 0.70	8882 ± 619	229 ± 20
Exposed	3.80 ± 0.62 (+14%)^{a}	3796 ± 360^{b}	130 ± 18^{b}
9.0 Month			
Control	7.68 ± 0.90	8947 ± 217	405 ± 15
Exposed	11.50 ± 1.21 (+50%)^{c}	3377 ± 186^{c}	163 ± 13^{c}

Number of Strain A/HEJ mice in each group = 10.
a = n.s.
b = $p < 0.05$.
c = $p < 0.0001$.

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